Investigation of the distribution of nitrite and nitrate and nitrite reductase activity in models of cardiovascular disease

Suborno Mukut Ghosh

Submitted in partial fulfillment of the requirements of the Degree of Doctor of Philosophy

University of London

Supervisor:

Professor Amrita Ahluwalia (QMUL)

Centre for Clinical Pharmacology

William Harvey Research Institute

Barts and the London School of Medicine and Dentistry

Charterhouse Square

London

EC1M 6BQ

Declaration of ownership

I declare that the results presented in this thesis are the result of my own work. All sources of information have been properly referenced and all help has been acknowledged.

Abstract

Recently, it has emerged that the NO metabolites, nitrite and nitrate can be chemically reduced in vivo to biologically active nitric oxide (NO). This generation of NO is dependent on reduction of nitrate to nitrite by facultative anaerobes on the dorsal surface of the tongue, entry of the nitrite into the enterosalivary circuit, transit to the stomach, and absorption through the gut wall into the circulation. Conversion of nitrite to NO is then facilitated by vascular nitrite reductase enzymes. This nitrate-nitrite-NO pathway has been shown to exert a number of beneficial effects in healthy volunteers e.g. lowering of blood pressure, however whether this pathway is affected by cardiovascular disease (CVD) is currently unknown.

Ozone chemiluminescence was used to determine and compare nitrite and nitrate levels in 2 models of CVD. To study atherosclerosis wild type (WT) and apolipoprotein E knock out (ApoE KO) mice were used and for hypertension wistar kyoto (WKY) rats as controls vs. spontaneously hypertensive rats (SHR). Assessment of nitrite reductase activity was conducted in the compartment which showed the most consistent differences in distribution, the red blood cell (RBC) and in homogenates of liver tissue. The impact of dietary nitrite and nitrate on distribution of the 2 anions throughout the cardiovascular system was assessed to determine the utility of this approach in restoring levels of these anions in CVD. Finally, using flow cytometry I investigated whether dietary nitrate supplementation could be used to influence inflammatory responses as a mechanism to improve CVD.

Compared to WT mice, nitrate levels were reduced in ApoE KO mice in the plasma and across most of the tissues. In contrast in SHRs, reduction of the anions was only apparent in RBCs with no differences compared to WKY in all other tested tissues. Furthermore I have demonstrated that the most efficient way to restore nitrate levels back up to baseline is through a dietary nitrate strategy and that a dose of 15mM nitrate in the drinking water is sufficient to achieve this. In addition I have shown that nitrite reductase activity is enhanced in CVD particularly at the level of the RBC in both

atherosclerosis and hypertension and that this enhanced activity is due, in part, to upregulation of xanthine oxidoreductase (XOR). Finally I have shown that dietary nitrate is an effective way to modulate an acute inflammatory response. This modulation is mediated through interfering with the ability of the neutrophil to firmly adhere to the vascular endothelium. These changes were shown to be dose-dependent and concomitant with dose-dependent increases in plasma nitrite and plasma nitrate. These data suggest that utilization of the nitrate-nitrite-NO pathway with dietary nitrate may represent an effective approach for the treatment of CVD.

Acknowledgements

As is often the case there are many people who need to be thanked for their support on completion of a PhD. I feel it is necessary to firstly thank the Medical Research Council for providing the funding for this research.

I would like to take this opportunity to thank my parents and brother. Since encouraging me and giving me the belief that I could actually do a PhD their unwavering support throughout my time in research have been invaluable.

Outside the research group: Dr. David Bishop-Bailey and Dr. Martin Carrier for acting as my progression panel. Many in Clin Pharm, in particular Fu and Kate as well as many outside of Clin Pharm but special mention must go to Emma, Chris, Michi, Thomas and in particular Giulia.

In the research group: Tara, Vanessa, Andy, Kristen, Manpreet, Kate, Lorna, Jasmine, Vikas, Daniel, Dan and Krishna. Special mention must be given to certain individuals including Amy (for making me cups of tea and providing me with a pick me up whenever I needed one) and Sven (for taking me out for cups of coffee and for generally being a good person to work with as well as being one of the most interesting people I have ever had the pleasure of knowing). Special mention must also go to Shanti for some particularly fond memories at the Atlanta and Pittsburgh conferences as well our holiday in the states. I could not have written this PhD without you as my sparring partner on those late nights and weekends distracting each other in the office ☺. Your support, encouragement and genuine kindness have been invaluable assets over the past few years.

I would like to thank Dr. Alex Milsom for being incredibly supportive during my first 2 years here. She taught me many things including how to do practical science well and accurately (most notably the technique of ozone chemiluminescence!), how to be

organized as well as instilling in me the desire to work hard and succeed. She was always there whenever I had questions and needed advice on aspects of work.

I would also like to thank Dr. Rayomand Khambata for so many things…some of them are written below, the rest will be discussed over a pint of London Pride. Our times together in the lab have been some of the most fun times I have ever had. Not only was it a pleasure working with him and learning so much from him (he really is a fountain of scientific (and F1!) knowledge) but it was a pleasure getting to know him and having a close friendship develop. Without him it would not have been possible to finish this PhD as his support, banter and encouragement have been invaluable during our time working together.

Finally and most importantly, I would like to thank my supervisor Professor Amrita Ahluwalia. Over the past 4 years her support and encouragement have been relentless. She has inspired me to push myself further than I ever thought possible and I have thoroughly enjoyed working for her. Not only has she provided me with so many opportunities to develop myself professionally as well as personally she has always been a source of positivity whenever results haven't gone as well as hoped. Her kindness and generosity is epitomized by the Christmas outings she has hosted for the group and her fun and enthusiastic spirit is best demonstrated by our weekly meetings where discussions have ranged from data and science to action films! It has been a real privilege for me to have worked for someone so dedicated to their work but also someone who genuinely cares about the people they work with. She is a rare character and I hope her success continues for many more years to come.

Publications

Inorganic nitrate ingestion improves vascular compliance but does not alter flow-mediated dilatation in healthy volunteers. Bahra M*, Kapil V*, Pearl S, **Ghosh S,** Ahluwalia A. *Nitric Oxide* 2012; 26: 197-202.

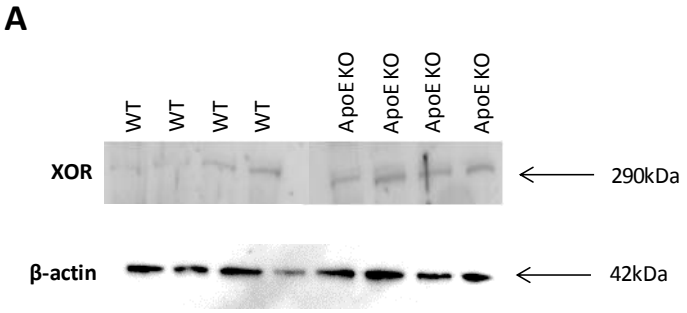
Dietary nitrate ameliorates pulmonary hypertension: cytoproctective role for endothelial nitric oxide synthase and xanthine oxidoreductase. Baliga RS, Milsom AB, **Ghosh SM**, Trinder SL, MacAllister RJ, Ahluwalia A, Hobbs AJ. *Circulation* 2012; 125: 2922-2932.

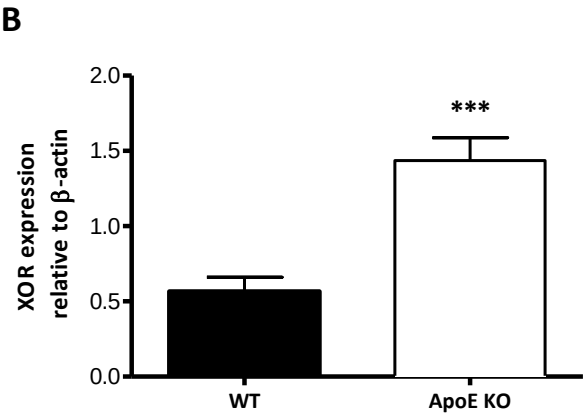
Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. **Ghosh SM***, Kapil V*, Fuentes-Calvo I*, Bubb KJ, Pearl V, Milsom AB, Khambata R, Maleki-Toyserkani S, Yousuf M, Benjamin N, Webb AJ, Caulfield MJ, Hobbs AJ, Ahluwalia A. *Hypertension* 2013; 61: 1091-1102.

Antiplatelet effects of dietary nitrate in healthy volunteers: involvement of cGMP and influence of sex. Velmurugan S, Kapil V, **Ghosh SM**, Davies S, McKnight A, Aboud Z, Khambata RS, Webb AJ, Poole A, Ahluwalia A. *Free radical biology and medicine* 2013; 65: 1521-1532

'Repurposing' of xanthine oxidoreductase as a nitrite reductase: a new paradigm for therapeutic targeting in hypertension. Khambata RS, **Ghosh SM**, Ahluwalia A. *Manuscript accepted by antioxidant and redox signalling* 2014.

Table of contents





List of figures

List of tables

Abbreviations

ADP	Adenosine 5’-diphosphate
AO	Aldehyde oxidase
ApoE	Apolipoprotein E
BH_4	Tetrahydrobiopterin
bp	Base pair
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CaM	Calmodulin
cGMP	Cyclic guanosine 3’5’-monophosphate
CVD	Cardiovascular disease
dNTP	Deoxynucleoside triphosphates
EC	Endothelial cell
ED	Endothelial dysfunction
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
g	Gravitational-force
G	Gauge
GAA	Glacial acetic acid
GTP	Guanosine 5’-triphosphate
Hb	Haemoglobin
H_2O	Water
HRP	Horse radish peroxidase
HTAB	Hexadecyltrimethylammonium bromide
ICAM-1	Intercellular adhesion molecule-1
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
IL-1β	Interleukin-1β
$K_3Fe(CN)_6$	Potassium ferricyanide
KI	Potassium iodide

KNO_2	Potassium nitrite
KNO_3	Potassium nitrate
KO	Knock out
L	Litre
min	Minute(s)
mARC	Mitochondrial amidoxime reducing component
MFI	Mean fluorescence intensity
$MgCl_2$	Magnesium chloride
MetHb	Methaemoglobin
Myoglobin	Mb
N_2	Nitrogen
NaF	Sodium fluoride
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
Na_3VO_4	Sodium orthovanadate
NEM	N-ethylmaleimide
Nb	Neuroglobin
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_3^-	Nitrate
NO_2^-	Nitrite
NO_2*	Excited nitrogen dioxide
NOS	Nitric oxide synthase
NOx	Nitrate + nitrite
NP-40	Nonidet P-40
O_2	Oxygen
O_3	Ozone
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
OxyHb	Oxyhaemoglobin
PCR	Polymerase chain reaction

RBC	Red blood cell
RFI	Relative fluorescence intensity
s	Second
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sGC	Soluble guanylate cyclase
SHR	Spontaneously hypertensive rat
SMC	Smooth muscle cell
ssDNA	Single stranded DNA
SO	Sulphite oxidase
TNFα	Tumour necrosis factor α
U	Units
UV	Ultraviolet
V	Voltage
VCl_3	Vanadium (III) chloride
vol	Volume
WBC	White blood cell
WKY	Wistar kyoto
wt	Weight
WT	Wild type
XO	Xanthine oxidase
XDH	Xanthine dehydrogenase
XOR	Xanthine oxidoreductase

Chapter 1:

Introduction

1.1 Preface

Cardiovascular disease (CVD) occurred most commonly in industrialized countries where the standard of living is particularly high in comparison to other nations, however in low- and middle-income countries (LMIC) CVD is rising. Globally, CVD is the main cause of death with approximately 17 million deaths occurring worldwide with over 80% taking place in LMICs (Chan, 2013) and over 4 million deaths occurring in Europe (Nichols *et al.*, 2012). In Europe, the UK is one of the worst affected countries with CVD accounting for almost 180,000 deaths with the majority (80,568) resulting from coronary heart disease (CHD). England alone accounts for almost 83% of these CVD-related deaths (Townsend *et al.*, 2012).

CVD is an umbrella term encompassing several diseases including CHD, stroke and myocardial infarction. A key risk factor for many CVDs is hypertension and it has been suggested that by 2027 30% of the population will likely present with hypertension. This is despite many available options in terms of pharmacotherapy (Law *et al.*, 2009). In addition a common underlying pathology for many CVDs, particularly stroke and myocardial infarction is atherosclerosis (Mendis *et al.*, 2011). The actual pathology for atherosclerosis is extremely complex. It involves all of the structural elements of the arterial wall, the circulating cells e.g. platelets and leukocytes, as well as a number of inflammatory cells in particular monocytes/macrophages (Lusis, 2000). Previously, atherosclerosis was considered to be the result of passive lipid accumulation in the vessel wall but the current reality is far more complex. In terms of therapeutics, whilst the introduction of angioplasty with stent insertion followed by anti-platelet therapy has resulted in substantial improvements in mortality following an event (Keeley *et al.*, 2006), limited options in primary or secondary prevention are available underlying the huge demands of increasing morbidity. Inorganic nitrate and nitrite via provision of nitric oxide (NO) may offer an option and is the focus of this thesis.

1.2 Overview of hypertension

The pathogenesis of hypertension is multifactorial, highly complex and much uncertainty still remains. Indeed, approximately 2-5% of patients have underlying renal

or adrenal disease as the cause of their raised blood pressure (Beevers *et al.*, 2001). For the remaining patients there is no single factor that is attributable for disease and in these particular circumstances the condition is termed essential hypertension. Essential, primary or idiopathic hypertension is defined as high blood pressure in which secondary causes such as renovascular disease, renal failure, aldosteronism or other causes of secondary hypertension are not present. Essential hypertension accounts for 95% of all causes of hypertension and is a heterogeneous disorder with different patients having various apparent causal factors that lead to high blood pressure (Carretero *et al.*, 2000). It is likely that a multitude of interrelated factors contribute to disease and the relative roles that these factors play differ from individual to individual. Salt intake, obesity, insulin resistance and the renin-angiotensin system are contributing factors that have gained much attention. Furthermore, additional contributors such as genetics and endothelial dysfunction are being evaluated (Beevers *et al.*, 2001).

The disease burden of arterial hypertension accounts for approximately 7.1 million deaths per year, which is equivalent to 13% of the total worldwide deaths (WHO, 2010). Indeed most cases of hypertension can be effectively treated with lifestyle changes, drugs or in some cases both, however within this population there is a cohort at one end of the spectrum i.e. those with hypertension that are resistant to treatment. Resistant hypertension has been defined as a raised blood pressure (seated clinic blood pressure >140/90mmHg) despite treatment with at least 3 antihypertensive agents at optimal or best tolerated doses. In the 2006 Health Survey for England, 20% of hypertensive patients had uncontrolled blood pressure despite the administration of at least 3 drugs, therefore in England alone it was estimated that 0.5-1 million people present with resistant hypertension (Falaschetti *et al.*, 2009), although recent estimates suggest that this 'resistance' may in part be accounted for by non-compliance. Indeed the recent assessment of global burden of disease (1990-2010) demonstrates that systemic hypertension remains the largest attributable risk factor for mortality worldwide (Lim *et al.*, 2010). In 2010 raised blood pressure was the second greatest and amongst the top 5 risk factors in the UK (Murray *et al.*, 2010) and

US (Murray, 2013) respectively. Worryingly, the scale of the problem is increasing, with the proportion of adults with hypertension predicted to increase to almost 1 in 3 (1.57 billion) by 2025 (Kearney *et al.*, 2005). Despite more than 60 years of innovation in the pharmacotherapy of hypertension (Laurent *et al.*, 2012), only ~25% of hypertensives are treated for their BP and of those treated only ~30-40% are controlled to guideline-driven targets (Falaschetti *et al.*, 2009; Egan *et al.*, 2010; Persell, 2011). Amongst the many facets linked to poor BP control, non-adherence to pharmacotherapy is commonly reported (Burnier *et al.*, 2013). A recent meta-analysis suggests that adherence (based upon prescription refill) to certain anti-hypertensive medications in primary prevention is ~50% (Naderi *et al.*, 2012), and direct measurement of this by spot urine testing has supported this view where of those patients categorised with resistant hypertension, 53% were non-adherent to their pharmacotherapy regimes (Jung *et al.*, 2013). Dislike of life-long pharmacotherapy, adverse medication effects and lack of hypertension symptoms are some of the reasons thought to underlie this non-adherence (Svensson *et al.*, 2000). Thus, therapeutic strategies that offer non-medication approaches are of great interest.

1.3 The basic aetiology of atherosclerosis

Atherosclerosis is considered a slowly progressing chronic inflammatory disease that results in the formation of lesions (plaques) in large and mid-sized arteries. Risk factors include hypertension, diabetes, smoking and excessive food intake. Previous infection (e.g. influenza, oral pathogens), underlying autoimmune diseases (e.g. lupus) or rheumatoid arthritis increase ones susceptibility for the development of atherosclerosis (Rosenfeld *et al.*, 2011; Gonzalez-Gay *et al.*, 2012; Sozeri *et al.*, 2012). Even though plaques can grow to sufficiently large size to compromise blood flow, most of its clinical complications are attributable to arterial occlusion due to plaque erosion or rupture (Lusis, 2000). Plaques form at predisposed regions characterized by disturbed blood flow such as curvatures and bifurcations i.e. branch points. When plaques are damaged and rupture, prothrombotic material from the plaque is exposed to the coagulation system in the blood resulting in sudden thrombotic occlusion of the artery at the site of disruption leading to inhibition of blood flow and ultimately an

event (Lusis, 2000). In the heart, atherosclerosis can lead to myocardial infarction and heart failure whereas in the arteries that perfuse the brain, it can cause ischaemic stroke and transient ischaemic attacks. If atherosclerosis affects other arterial branches, renal impairment, hypertension, abdominal aortic aneurysms and critical limb ischaemia may result (Mendis *et al.*, 2011).

1.4 Atherosclerosis as a disease of inflammation

The notion of atherosclerosis as an inflammatory disease is based on the finding that immune competent cells are abundant in atherosclerotic lesions, and that lesions are also sites for cytokine synthesis, in particular pro-inflammatory cytokines (Frostegard *et al.*, 1999). Cells of both the innate and the adaptive immune system play a crucial role in atherosclerosis development. Through the transformation of immune cells into pro- and anti-inflammatory chemokine- and cytokine-producing cells as well as guiding the interactions between the different immune cells, the immune system critically influences the tendency of a plaque to rupture and cause clinical symptoms such as myocardial infarction and stroke.

The development of atherosclerosis is initiated by activation, dysfunction and structural alterations of the endothelium, which is critical to the pathology of atherosclerosis. Activated endothelium with the expression of adhesion molecules is an early event in atherosclerosis (Drexler *et al.*, 1991; Celermajer *et al.*, 1992; Blankenberg *et al.*, 2003; Halcox *et al.*, 2009). Adhesion molecule expression allows mononuclear leukocytes, such as monocytes and T-cells, to attach to the endothelium and penetrate into the intima. Dendritic cells, mast cells, neutrophils, B-cells, smooth muscle cells (SMCs) may also be present in lesions. Contractile SMCs undergo phenotypic changes into synthetic SMCs and migrate into the intima from the media (Weber *et al.*, 2011).

The vascular endothelium essentially acts as a dynamic interface/barrier between the circulation and the arterial wall. The artery wall changes throughout ones life but the process of atherosclerosis is considered to be different from ageing. The inflammatory

nature of atherosclerosis and therefore CVD has been accepted for a long time. However, even with this understanding of the disease process there is no anti-inflammatory or immune modulatory treatment available to ameliorate disease. The high incidence of atherosclerosis-related CVDs naturally causes an enormous and sustained burden on healthcare systems (Weber *et al.*, 2011). Current therapeutics largely target alleviating hypertension and hyperlipidemia or controlling haemostasis to prevent thrombotic complications. Furthermore lifestyle changes can be implemented e.g. eating a healthier diet, exercising more, and use of medications such as cholesterol-lowering statins can be used in the treatment of atherosclerosis. In some cases surgery may be required to widen or bypass a section of a blocked or narrow artery. Unfortunately these strategies do not directly address the inflammatory processes that drive disease progression (Weber *et al.*, 2011).

As the immune response is a highly complex system numerous subtle targets for therapeutic manipulation are offered. As research uncovers the specifics of the host response to atherosclerosis more selective interventions might prove more appropriate for longer term atherosclerosis treatment. Clearly strategies that look to modulate the inflammatory response in atherosclerosis have potential therapeutic utility.

1.5 The healthy endothelium

In 1966 (Florey, 1966) it was suggested that vascular endothelium is more than a simple monolayer of cells and in the last few decades the importance of the endothelium in the regulation of cardiovascular physiology has been uncovered. The endothelial cell surface in an adult human is comprised of approximately 1 to $6x10^{13}$ cells, weighs approximately 1kg and covers a surface area of approximately 1 to $7m^2$ (Augustin *et al.*, 1994). The healthy endothelium regulates vascular tone and structure as well as exhibiting anticoagulant, antiplatelet and fibrinolytic properties. The maintenance of these effects is accomplished by the release of numerous substances (Villar *et al.*, 2006; Roberts *et al.*, 2013). In the healthy endothelium there are 3 main substances thought to provide many of the beneficial properties of the endothelium

called endothelium-derived relaxing factors (EDRFs) that include prostaglandins, endothelium-derived hyperpolarizing factor (EDHF) (Chen *et al.*, 1988) and nitric oxide (NO) (Furchgott *et al.*, 1980), which is possibly the most important of all of the EDRFs (figure 1.1).

These beneficial factors are released by the endothelium in response to physiological forces such as shear stress (Sessa *et al.*, 1994) and by recirculation of locally generated hormones including bradykinin (BK), adenosine and vascular endothelial growth factor (VEGF) (Govers *et al.*, 2001) (figure 1.1). NO (discussed in section 1.6) generated from the amino acid substrate L-arginine by the action of endothelial nitric oxide synthase (eNOS), is freely able to diffuse and crosses the underlying vascular SMCs as well as pass into the lumen where it acts upon platelets and leukocytes. It produces most of its effects in target cells by activating soluble guanylate cyclase (sGC), which leads to elevations in cyclic guanosine monophosphate (cGMP).

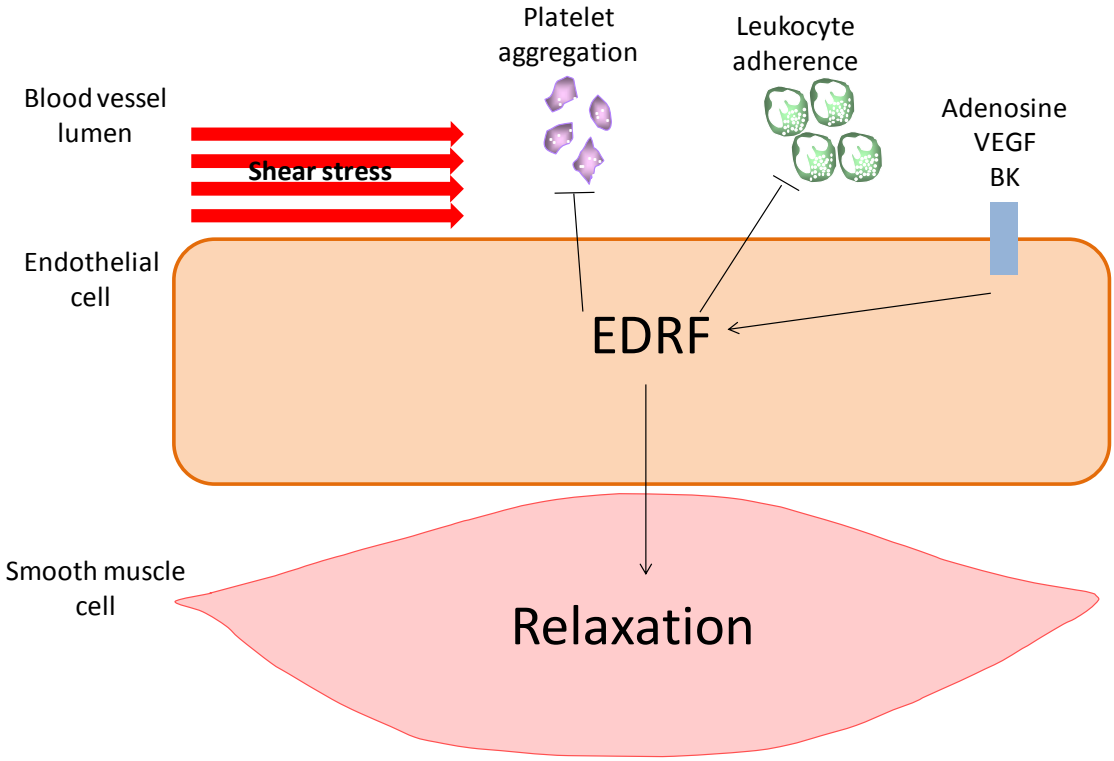


Figure 1.1: Production and release of EDRF from the endothelium to promote smooth muscle cell relaxation and exert anti-platelet and anti-inflammatory properties in the blood vessel lumen. BK, bradykinin; EDRF, endothelium-derived relaxing factor; VEGF, vascular endothelial growth factor.

EDHF activity is most well defined with respect to its hyperpolarizing effect on vascular SMCs resulting in potassium conductance and subsequent relaxation of vascular SMCs thus playing a critical role in maintaining vascular tone (Busse *et al.*, 2002). The identity of EDHF is uncertain and may differ between vascular beds. It is however, well recognized that EDHF can compensate for loss of NO-mediated vasodilator tone in certain disease states including hypertension (Taddei *et al.*, 1994), particularly in the microcirculation, and this appears important when NO bioavailability is reduced (Halcox *et al.*, 2001). More recently there has been the suggestion that similarly to NO, EDHF might also influence inflammatory cell recruitment (Villar *et al.*, 2011) and that EDHF pathways may play a greater role in determining vascular health in females compared to males (Scotland *et al.*, 2005; Bubb *et al.*, 2012).

Prostacyclin, generated from arachidonic acid by the action of the cyclooxygenase enzymes, is another endothelium-derived mediator that expresses many characteristics displayed by NO (Moncada *et al.*, 1977). The effects of prostacyclin are dependent upon the expression of its receptor, the prostacyclin receptor (IP) and consequent activation of adenylate cyclase. Prostacyclin is a dilator that inhibits platelet activation (Mustard *et al.*, 1980) and some evidence supports a role in reducing leukocyte activation (Lefer *et al.*, 1994; Thomson *et al.*, 1994). Figure 1.2 details the 3 main endothelial factors and their pathways, thought to underlie normal vascular health.

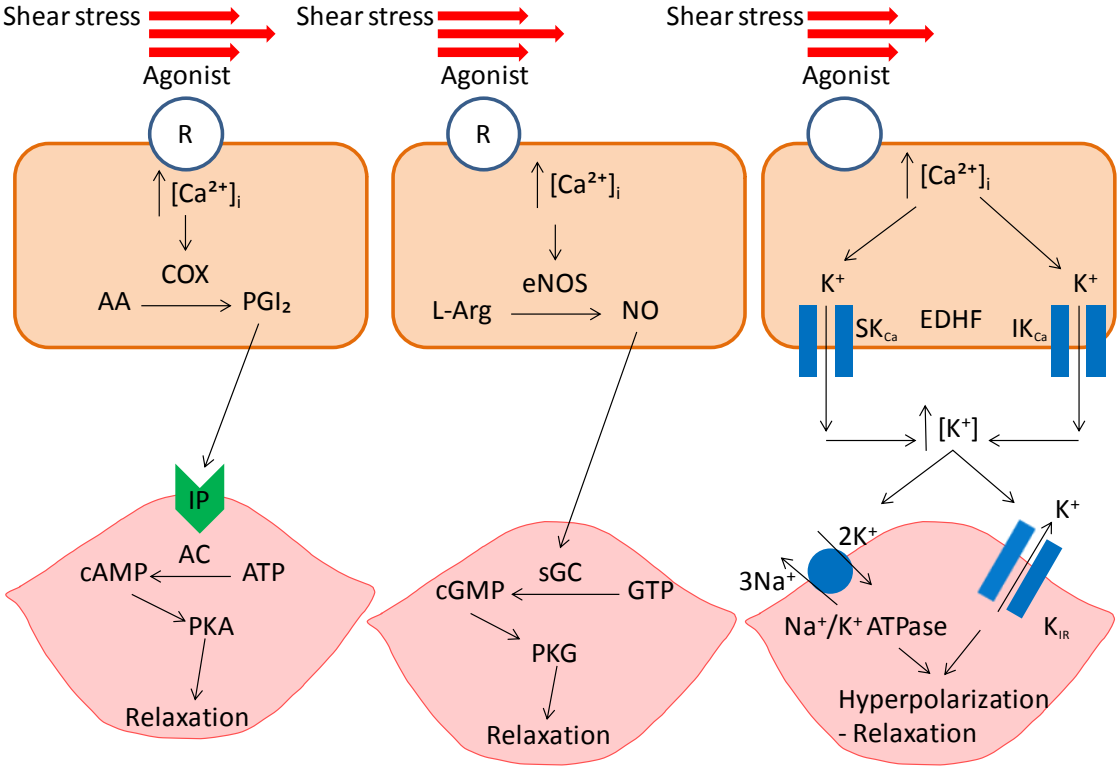


Figure 1.2: Schematic summarizing the release of relaxing factors from endothelial cells and their effect on vascular SMCs. AA, arachidonic acid; ATP, adenosine triphosphate; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; Cox, cyclooxygenase; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; GTP, guanosine triphosphate; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+ channel; IP, IP receptor; K^+, potassium; L-Arg, L-arginine; Na^+, sodium; NO, nitric oxide; PGI_2, prostacyclin; PKA, protein kinase A; PKG, protein kinase G; sGC, soluble guanylate cyclase; SK_{Ca}, small conductance Ca^{2+}-activated K^+ channel.

Critically in CVD there is endothelial dysfunction. This dysfunction, however, represents not only a deficit of the bioavailability of the aforementioned mediators but also expression of damaging factors. For example, evidence demonstrates the generation of the potent constrictor and pro-hypertrophic mediator endothelin-1 as well as a switch from the generation of vasoprotective prostacyclin to damaging prostanoids including prostaglandin E_2 (PGE_2), and enhanced conversion of angiotensin I to angiotensin II (Saye *et al.*, 1984; Kinlay *et al.*, 2001). These changes underlie the switch in phenotype of the endothelium from beneficial to damaging. Perhaps the most prominent and well described change is the reduction in bioavailable NO. Indeed a loss in NO-mediated dilation (induced by shear stress) is used to characterize endothelial dysfunction (Celermajer *et al.*, 1992; Celermajer *et al.*, 1994).

1.6 Nitric oxide (NO)

Nitric oxide (NO) was discovered in 1772 by Joseph Priestley as a clear, colourless gas with a half-life of 6-10 seconds (Priestley *et al.*, 1772). In 1977 it was shown that sodium nitroprusside, nitroglycerin, sodium azide and hydroxylamine all increased guanylate cyclase activity in particulate and/or soluble preparations from various tissues (Katsuki *et al.*, 1977). Activation of guanylate cyclase by these compounds consequently resulted in increases in cGMP levels. At the time the precise mechanism of activation was unknown, however it was postulated that activation may be due to the formation of NO since NO also increased guanylate cyclase activity (Katsuki *et al.*, 1977).

It wasn't until 1979 that the vascular smooth muscle relaxant properties of NO were discovered. Using bovine arterial strips in an organ bath Gruetter saw that the carcinogenic nitrosamine also known to liberate NO 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), nitroprusside and NO all caused concentration-dependent relaxation of precontracted strips (Gruetter *et al.*, 1979). Relaxation in response to NO was rapid but returned spontaneously to the initial level of induced tone. Coronary arterial relaxation by NO was abolished by agents that attenuated NO reactivity such as haemoglobin, methaemoglobin, myoglobin and the oxidant methylene blue (Gruetter *et al.*, 1979).

In 1980, Furchgott discovered that endothelial cells produce EDRF in response to stimulation by acetylcholine (ACh) in vessels with intact endothelium. In this study it was demonstrated that relaxation of isolated preparations of rabbit thoracic aorta, and other blood vessels, by ACh required the presence of an intact endothelium as disruption of the integrity of the endothelium (through rubbing it) caused a loss of ACh-dependent relaxation (Furchgott *et al.*, 1980). ACh acting on muscarinic receptors of the endothelial cells stimulated release of a substance (or substances), which in turn acted on the SMCs in the media to activate relaxation. At the time, the substance was not identified to be NO, however other candidates such as bradykinin, prostacyclin and AMP were all ruled out (Furchgott *et al.*, 1980).

In 1987, separately, Moncada et al. and Ignarro et al. proved that EDRF is NO. Prior to the discovery of EDRF as NO it was actually suggested by Furchgott that EDRF may be NO. Moncada et al. examined this suggestion by studying the release of EDRF and NO from endothelial cells in culture (Palmer *et al.*, 1987). NO was determined by chemiluminescence and the biological activity of EDRF and NO was determined using bioassay tissues. The relaxations of bioassay tissues induced by EDRF and NO were indistinguishable. Bradykinin caused concentration-dependent release of amounts of NO sufficient to account for the relaxations that were induced by EDRF (Palmer *et al.*, 1987). In the study by Ignarro et al. similar patterns were seen in that the effects of EDRF were indistinguishable to the effects produced by NO. Their study, however, looked at the vascular effects of EDRF released from perfused bovine intrapulmonary artery and vein and subsequently compared them to the effects of NO delivered by superfusion and over endothelium-denuded arterial and venous strips arranged in a cascade (Ignarro *et al.*, 1987).

A year later, Moncada demonstrated that NO is synthesized from the amino acid L-arginine in porcine aortic endothelial cells in culture. In this study, NO detection was achieved by bioassay, chemiluminescence and mass spectrometry. Release of NO from the endothelial cells was induced by bradykinin or the calcium ionophore A23187 (Palmer *et al.*, 1988a). Infusions of L-arginine and L-citrulline but not D-arginine (or other close structural analogues) reversibly enhanced the release of NO. Finally using ^{15}N-labelled L-arginine, mass spectrometry analysis indicated that the enhanced release of NO was due to the formation of NO from the terminal guanidine nitrogen atom(s) of L-arginine (Palmer *et al.*, 1988a). As the importance of L-arginine in the generation of NO was determined this opened up opportunities for investigation of the pathways of the synthesis of NO.

1.7 Nitric oxide synthase (NOS) family of enzymes

The enzymatic production of NO from L-arginine is catalysed by 1 of 3 nitric oxide synthases (NOS) in a 2 step process via the formation of N-hydroxyl L-arginine (Dawson *et al.*, 1994). NO has a multitude of functions throughout the body and 3 different

enzymes exist to tailor for its different locations and functions. The enzymes are all the same with respect to the fact that they all produce NO, however differences in structure and location allowing them to cater for the various functions of NO (the similarities and differences are summarized in table 1.1).

The 3 isoforms have been characterized, purified and cloned in the order of their discovery: neuronal nitric oxide synthase (nNOS) or NOS1, inducible nitric oxide synthase (iNOS) or NOS2 and endothelial nitric oxide synthase (eNOS) or NOS3. The 3 isoforms are actually encoded by 3 different genes on different chromosomes although they share between 50-60% homology in the amino acid sequence and in the amino acid sequence in the oxidase and reductase domains (Marletta, 1994; Govers *et al.*, 2001).

The first isoform isolated of NOS was reported in the brain. Initially the authors showed that nitric oxide synthase required CaM (calmodulin) as NO formation required calcium. They observed that CaM restored unrecovered enzymatic activity (in diethylaminoethyl eluate fractions). Furthermore it was shown that NO formation was dependent on the conversion of arginine to citrulline (Bredt *et al.*, 1990). This was done by utilizing an assay that monitors the conversion of [^{3}H]arginine to [^{3}H]citrulline. Finally, based on the discovery that enzymatic activity was CaM-dependent and converted arginine to citrulline, it was possible to purify NO synthase to homogeneity from rat cerebellum. The purified enzyme was shown to exist as a monomer 150kDa in size (Bredt *et al.*, 1990) and was termed neuronal NOS (nNOS).

Soon after the discovery of nNOS, iNOS was isolated from macrophages. In a 2-column procedure using an affinity column and anion exchange column iNOS was purified from activated murine macrophages. Enzymatic activity was dependent on L-arginine and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Hevel *et al.*, 1991). Moreover factors such as tetrahydrobiopterin (BH_4) and flavin adenine dinucleotide (FAD) enhanced activity whereas magnesium and flavin mononucleotide (FMN) had no effect. The molecular weight of the protein was estimated to be

260±30kDa suggesting that the enzyme exists as a dimer (Hevel *et al.*, 1991). iNOS is an enzyme that can be found in a number of cell types normally after several hours of exposure to cytokines and/or microbial products. The final isomer of NOS identified was endothelial NOS (eNOS) described in section 1.10.

	NOS1/NOS3	**NOS2**
Ca^{2+}/calmodulin-dependent	√	x
Constitutive/inducible	constitutive	inducible
Cytosolic	√	√
Period of NO release	short	long
Amount of NO released	picomoles	nanomoles
Type of stimulation	receptor/physical	induced after macrophage activation, endothelial cells and a number of other cytokines
Induction inhibited by glucocorticoids	x	√
Role	transduction mechanism	effector molecule whose release is induced during immunological reactions

Table 1.1: Summary of the similarities, differences and differing functions of the 3 NOS isoforms

1.8 Overview of NOS inhibitors

In the literature numerous NOS inhibitors have been described. Inhibitors of NOS have been described which interact with the NOS enzymes in a variety of ways, for example at different sites, as well as different time- and substrate-dependence and mechanism of inhibition. Of these the most widely used have been NG-monomethyl L-arginine (L-NMMA), N-nitro L-arginine (L-NNA) and N-nitro L-arginine methyl ester (L-NAME). Simple arginine derivatives were first considered as inhibitors for experimental use because they were expected to compete with arginine for the active site of NOS. L-NMMA occurs naturally in living organisms as it is the product of the degradation of arginine-methylated proteins (Bedford *et al.*, 2009). It is one of the first compounds which were intuitively employed to inhibit NOS's at the end of the 80s (Palmer *et al.*, 1988b). L-NMMA has been widely used as a tool to decrease NO bioavailability or to establish the NO dependency of a physiological process. As the structure of L-NMMA is very close to arginine it acts as a competitive inhibitor of all NOS enzymes (Griffith *et*

al., 1996). An additional feature of L-NMMA is that it is easily taken up into cells via the action of cationic amino acid transporters since it has a similar affinity to arginine transport systems as L-arginine (McDonald *et al.*, 1997).

L-NNA was one of the first synthetic NOS inhibitors produced being demonstrated to block NO synthesis in the early 90s (Moore *et al.*, 1990). Initially L-NNA was thought to act as a competitive inhibitor of all NOS isoforms with high selectivity for eNOS and nNOS rather than iNOS (Furfine *et al.*, 1993). However a later study indicated only minor selectivity to nNOS and eNOS (Moore *et al.*, 1996). Such a pattern of selectivity correlates with a different mechanism of L-NNA binding to individual isoforms. The binding of L-NNA to eNOS and nNOS is a time-dependent process with a relatively slow reversal (Furfine *et al.*, 1994; Klatt *et al.*, 1994). The only limiting factor of L-NNA application in biological systems is poor solubility (approximately 4mmol/L) at neutral pH. This issue was addressed by introducing L-NAME. L-NAME may act as a weak NOS inhibitor, however it is readily hydrolyzed by ubiquitously present esterases to L-NNA in a biological system (Griffith *et al.*, 1996). The application of L-NAME in experiments in vitro and in vivo provides an advantage over L-NNA as there is no major limitation on solubility in an aqueous environment.

The vast majority of this work contributed to the establishment of NO as a critical signalling molecule in the cardiovascular system. By 1993, NO had been implicated in the pathogenesis of diseases ranging from hypertension to septic shock (Moncada *et al.*, 1993) and it is the wide-ranging importance of this molecule that ultimately led to the Nobel prize in Physiology or Medicine in 1998 being awarded to Robert Furchgott, Louis Ignarro and Ferid Murad. Over the last 2 decades NO research has crossed into almost every area of biomedicine that as such it is now hard to find a physiological system in which NO does not play a regulatory role. The wide biological significance of NO comes from the fact that it is widely synthesized but also because it is a very reactive molecule.

1.9 Endothelial NOS (eNOS)

eNOS was purified from cultured and primary bovine aortic endothelial cells. The resultant protein was approximately 135kDa in size (Pollock *et al.*, 1991). The activity of the enzyme was confirmed through its effect on sGC of rat fetal lung fibroblasts by measuring nitrite/nitrate formation and by bioassay on endothelium-denuded vascular strips. Furthermore activity was shown to be dependent on L-arginine, NADPH, calcium (Ca^{2+}), CaM and BH_4 (Pollock *et al.*, 1991) as with other isoforms.

We now know that the 3 NOS isoforms are homodimeric proteins that require L-arginine as the substrate as well as molecular oxygen and NADPH as co-substrates as well as FAD, FMN and BH_4 as essential cofactors (Marletta, 1994). Between the 3 of them the NOS enzymes cover a range of functions but of particular relevance to the cardiovascular system is eNOS as it is the predominant isoform in the vasculature and is therefore responsible for most of the NO produced within this system.

1.9.1 The structure of eNOS

eNOS is composed of 2 globular protein modules (reductase and oxygenase domains) connected via a flexible protein strand. The C-terminal reductase domain contains binding sites for NADPH, FMN and FAD whereas the N-terminal oxygenase domain, which subtracts 1 electron from L-arginine, has binding sites for heme iron, BH_4 and L-arginine (see figure 1.3 for the structure of eNOS) (Knowles *et al.*, 1994). From the carboxy-terminal reductase domain a normal functioning NOS enzyme generates the electrons required for NO synthesis by binding reduced NADPH and catalyzing its dehydrogenation. The electrons are transferred across the flexible protein strand from NADPH, via FAD and FMN, to the haem in the amino-terminal oxygenase domain. The electron transfer is activated by calcium-dependent binding of CaM to a specific binding site on the flexible protein strand (Knowles *et al.*, 1994). The oxygenase domain consists of the catalytic center responsible for NO production. At the oxygenase domain the cofactors BH_4, molecular oxygen and L-arginine can also be bound. At the haem site of the oxygenase domain electrons are used to reduce and

activate oxygen and to oxidize L-arginine to L-citrulline and NO (Knowles *et al.*, 1994; Forstermann *et al.*, 2006; Dias *et al.*, 2009).

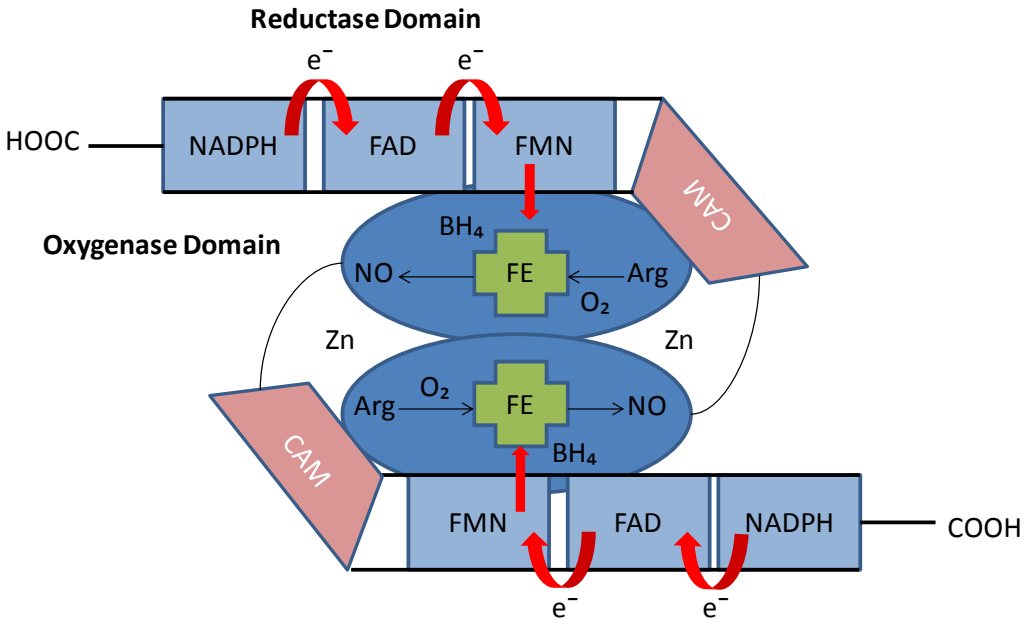


Figure 1.3: A model for the dimeric structure of eNOS. Electron (e^-) transfer between cofactors and substrates is represented by the red arrows. The electrons flow from NADPH to FAD to FMN in the reductase domain of 1 monomer to iron (Fe) in the oxygenase domain of the adjacent monomer. Electron flow and catalysis is shown for both sides of the enzyme. Electron flow from 1 domain to the other is mediated by calmodulin (CAM) (adapted from (Munzel *et al.*, 2005b)).

1.9.2 Biochemistry of eNOS-derived NO

eNOS is mostly expressed in endothelial cells but its expression is not restricted to this cell type. Indeed eNOS has been detected in cardiac myocytes, platelets and certain neurons of the brain (Forstermann *et al.*, 1994). Importantly eNOS (as well as nNOS) is inactive at basal intracellular Ca^{2+} concentrations and requires an increase in intracellular Ca^{2+}, through stimulation by hemodynamic shear stress or by the activation of G-protein coupled cell surface receptors to trigger activation.

eNOS is usually found in regions of the plasma membrane called caveolae (small invaginations of the plasma membrane). Caveolae are structures associated with compartmentalizing signal transduction molecules that regulate endothelial cell functions including the generation of NO by eNOS (Drab *et al.*, 2001). The initial step in NO production requires localization of eNOS to caveolae and requires cotranslational

myristoylation as well as posttranslational palmitoylation of eNOS (Liu *et al.*, 1995). Only a fraction of eNOS is sequestered in the caveolae and it is this fraction that can interact with the caveolae protein caveolin-1. Caveolin-1 (a major coat protein of caveolae) is critical in regulating enzymic function as it tonically inhibits eNOS function by directly interacting with the enzyme (Garcia-Cardena *et al.*, 1997). Indeed in caveolin-1 gene disrupted mice, blood vessels show enhanced endothelium-dependent relaxation (Drab *et al.*, 2001). Displacement and therefore interruption of caveolin-1 interaction with eNOS leads to activation of eNOS.

As with nNOS, calcium-activated CaM is one of the key pathways for the regulation of eNOS activity. An increase in intracellular Ca^{2+} leads to enhanced binding of CaM to the enzyme (Forstermann *et al.*, 1991; Pollock *et al.*, 1991). Binding of CaM to its specific binding site increases the rate of electron transfer from the reductase domain to the catalytic centre of eNOS. The enhanced binding dislodges the auto-inhibitory loop leading to the facilitation of electron flow from NADPH (located in the reductase domain) to haem (located in the oxygenase domain) (Forstermann *et al.*, 2012).

eNOS activity is also regulated by phosphorylation. eNOS can be phosphorylated on several serine (Ser), threonine (Thr) and tyrosine (Tyr) residues. There are 2 critically established, functionally important phosphorylation sites in human eNOS, namely Ser1177 and Thr495 (Fleming *et al.*, 2001). In a resting endothelial cell Ser1177 is not normally phosphorylated. Exposure to oestrogens, VEGF, insulin, bradykinin or fluid shear stress can induce phosphorylation (Michel *et al.*, 1993; Papapetropoulos *et al.*, 1997; Dimmeler *et al.*, 1999; Fulton *et al.*, 1999). This activating phosphorylation requires recruitment of the kinase Akt and of heat shock protein 90 (Hsp90) (Garcia-Cardena *et al.*, 1998). Hsp90 functions as a scaffold for eNOS and Akt. Conversely phosphorylation at Thr495 inactivates eNOS (Fleming *et al.*, 2001; Fleming *et al.*, 2003).

BH_4 is a required cofactor of NO synthesis as it is a critical determinant of eNOS activity. Importantly depletion of BH_4 may result in 'uncoupling' (see figure 1.4) of

eNOS with a consequent reduction in NO synthesis and production of superoxide instead of NO by eNOS. Even when NO is produced from the healthy endothelium it can be rapidly inactivated by superoxide, particularly under conditions of high oxidant stress (Alp *et al.*, 2004).

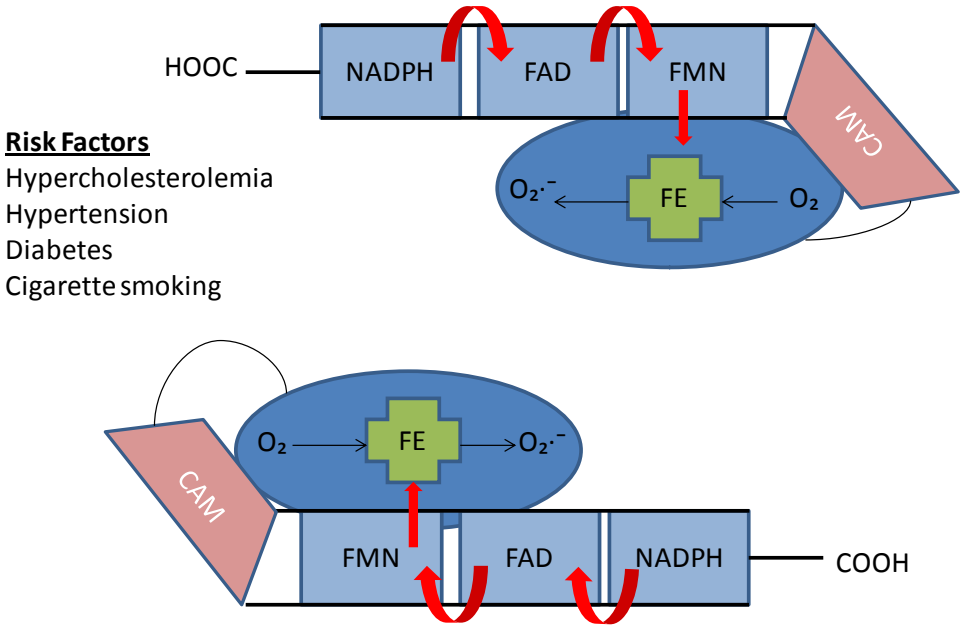


Figure 1.4: Potential risk factors that may cause depletion of BH_4 ultimately leading to eNOS uncoupling and production of superoxide ($O_2 \cdot^-$) and potentially endothelial dysfunction. In many types of vascular disease, products of vascular disease (such as peroxynitrite) combine to oxidize BH_4, the essential cofactor of eNOS and/or produces damage to eNOS. Subsequently, O_2 reduction by eNOS is uncoupled from NO formation and a functional NOS is converted into a dysfunctional $O_2 \cdot^-$ generating enzyme that contributes to vascular oxidative stress (adapted from (Munzel *et al.*, 2005b).

1.10 Biochemistry of NO reactions

The steady-state concentration of NO is determined by its rate of formation and its rate of decomposition. The metabolic pathway of NO depends strongly on its site of administration or formation. In biological systems, the mode and rate of NO metabolism is dependent on its own concentration, its diffusibility and the surrounding concentration of other bioreactants. Diffusion is essential for understanding the ability of NO to act as a local modulator and is a major determinant of biological life time (Lancaster, 1994; Beckman *et al.*, 1996a). In the absence of haemoglobin, NO rapidly diffuses along the vasculature and reaches vascular SMCs and mitochondria of cardiomyocytes in sufficiently bioactive amounts to affect coronary blood flow and left

ventricular function (Kelm *et al.*, 1995; Kelm *et al.*, 1997). Within the myocardial tissue, endothelial NO is avidly bound to myoglobin, resulting in steady-state concentration of 0.1-0.3μM, which exerts inhibitory effects on cytochrome oxidase and the electron transfer of the respiratory chain in the mitochondria (Poderoso *et al.*, 1998). Thus in agreement with its function as a paracrine mediator, NO can travel significant distances to reach target cells neighbouring the NO generating cell with little consumption or direct reaction. Along this migration, in particular at higher concentration, NO can interact with molecular oxygen, thiols and reduced hemoproteins. The extent of either of these reactions largely depends on the microenvironmental conditions in which NO is released, most important, the concentration of other bioreactants (Heinrich *et al.*, 2013).

Aside from molecular oxygen, several reactive oxygen derived species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$) interact with NO. The main biological targets for NO within the cell are oxygen-centred radicals, metal-, thiol- and amine containing proteins and low molecular weight thiols. Many of these targets are biologically active and are involved with a diverse array of normal cellular and physiological processes. Although the chemical reactions in which NO participate are quite varied, the constraints imposed by biological conditions narrow the relevant reactions considerably. It is possible to view these reactions as either direct or indirect reactions of NO (Wink *et al.*, 1996; Thomas *et al.*, 2008).

Direct reactions refer to those interactions in which NO binds directly with biological targets, predominantly metal complexes, radical species and oxygen. These reactions are generally rapid, require low concentrations of NO and are the basis for the majority of NO-related biological effects. The reaction of NO with certain metals to form nitrosyl complexes occurs in vivo mainly with iron-containing proteins (Thomas *et al.*, 2003). The reaction between NO and haem proteins represents a critical signaling pathway, the most well studied example being NO's interaction with sGC, a key enzyme in the pathway of NO-mediated vascular dilation (Denninger *et al.*, 1999; Munzel *et al.*, 2003) and discussed later. The reaction between NO and haem proteins

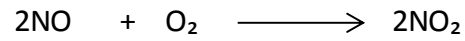
represents an important signaling pathway which is influenced by the redox state of the haem and the structure of the protein. For example, in terms of redox state, the rate constant for NO dissociation from a ferric (iron (III), Fe^{3+}) haem protein is 4 times that of the corresponding ferrous (iron (II), Fe^{2+}) haem (Kharitonov *et al.*, 1997; Sharma *et al.*, 1999). Ferrous haem also has a comparably faster association rate, making these complexes relatively stable and ferric haem protein complexes reversible in many cases (Ford *et al.*, 2002; Thomas *et al.*, 2003). The most well studied example of NO interaction with haem is the reaction with sGC. sGC is an obligate heterodimer consisting of 1 α and 1 β subunit and a non-covalently bound haem (Wedel *et al.*, 1995). More than 1 model of sGC activation/deactivation have been suggested (Poulos, 2006), however it is generally agreed that nitrosylation of the haem severs the proximal histidine bond, triggering intramolecular rearrangements which influence the catalytic centre and result in a significant increase in cGMP levels (Ignarro *et al.*, 1982; Zhao *et al.*, 1999). The severing of the histidine bond converts the haem from an initial 6-coordinate Fe^{2+}-nitrosyl intermediate to a 5-coordinate Fe^{2+}-nitrosyl species (Stone *et al.*, 1994; Stone *et al.*, 1996; Ballou *et al.*, 2002), a process that may be accelerated by additional non-haem NO (Cary *et al.*, 2006). Increased levels of cGMP resulting from sGC activation decrease the intracellular calcium concentration resulting in dephosphorylation of myosin light chains and subsequent relaxation of vascular smooth muscle, eliciting the classic pathway of NO-dependent vasodilation (Munzel *et al.*, 2003). Other NO-mediated nitrosylation pathways also inhibit metalloproteins such as cytochrome P-450, NOS, cytochrome oxidase and catalase (Wink *et al.*, 1997).

Furthermore, NO can react directly with high energy radicals such as carbon-, oxygen- and nitrogen-centred radicals. Arguably the most significant reaction is with superoxide (O_2^-, formed from the 1 electron reduction of molecular oxygen) forming the nitrogen oxide peroxynitrite ($ONOO^-$, equation 1.1) (Beckman *et al.*, 1990).

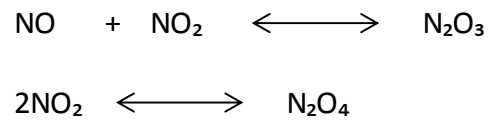
$$NO + O_2^- \longrightarrow ONOO^-$$

Equation 1.1: The reaction of nitric oxide (NO) with superoxide (O_2^-) to generate peroxynitrite ($ONOO^-$).

Peroxynitrite can cause severe tissue damage through its reactions with proteins, lipids and DNA (Szabo *et al.*, 2007). Peroxynitrite decays rapidly once protonated forming peroxynitrous acid (ONOOH) and eventually nitrate (Beckman *et al.*, 1990). This interaction of NO with superoxide is thought to play a critical role in the phenomenon of endothelial dysfunction (Channon *et al.*, 2002). The reaction between NO and oxygen (commonly referred to as autoxidation) forms nitrogen dioxide (NO_2, equation 1.2) and other nitrogen oxides such as N_2O_3 and N_2O_4 (termed reactive nitrogen oxide species, equation 1.3).



Equation 1.2: The autoxidation reaction of nitric oxide (NO) with oxygen (O_2) forming nitrogen dioxide (NO_2).



Equation 1.3: The formation of other nitrogen oxides (N_2O_3 and N_2O_4) from the reaction of NO with NO_2.

The autoxidation reaction is second order with respect to NO and first order with respect to oxygen and therefore third order overall and involves 2 NO molecules with 1 oxygen (equation 1.2). The reaction is relatively slow at physiological NO concentrations in health but is more relevant in certain environments such as in the immediate vicinity of NO production or activated macrophages and neutrophils or under pathophysiological conditions where the NO concentration is raised.

The indirect reactions of NO are often mediated by products of the direct reactions in particular reactive nitrogen oxide species derived from the interactions of NO with molecular oxygen or superoxide. It is the reactive intermediates and not NO itself that ultimately react with target specific motifs. Indirect reactions tend to require initial higher concentrations of NO due to the lower relative concentrations of the reactants. Nitrogen oxides range from the fully oxidized nitrate (NO_3^-) to the fully reduce

ammonia (NH_3) and in between these 2 states exists a range of partially reduced nitrogen oxides (Wink *et al.*, 1996; Thomas *et al.*, 2008).

In the vascular system on production in the endothelium, NO diffuses into the underlying smooth muscle and reacts directly with sGC to elicit vasodilation. NO diffusing into the blood stream is rapidly metabolized to various reaction products. In blood the formation of NO metabolites is dependent on the environmental conditions at a given moment. Key factors that require consideration are the concentration of NO itself, the surrounding conditions (e.g. partial pressure of oxygen, pH, and type and amount of free radicals), concentration of thiols and the presence of haem proteins (e.g. guanylate cyclase, haemoglobin, superoxide dismutase and xanthine oxidoreductase) (Stamler *et al.*, 1996; Kelm, 1999). In the plasma compartment autoxidation of NO produces NO_2 (equation 1.2), which can either dimerise to form N_2O_4 or couple with unreacted NO to form N_2O_3 (equation 1.3) (Stamler *et al.*, 1996). These nitrogen oxides then either nitrosate low molecular weight thiols including cysteine, thiol groups, amines to produce N-nitroamines or they can be further hydrolysed to produce nitrite (NO_2^-) and nitrate (NO_3^-) (Lewis *et al.*, 1994). Due to the concentration of reactants the primary pathway through which NO is metabolized in blood is reaction with the haem group of oxy- and deoxyhaemoglobin contained in the red blood cells (RBCs). The reaction between NO and oxygenated haemoglobin forms nitrate and methaemoglobin (equation 1.4).

$$NO + HbO_2 \longrightarrow MetHb + NO_3^-$$

Equation 1.4: The primary reaction pathway of NO in blood involves reaction with oxygenated haemoglobin (HbO_2) to generate methaemoglobin (MetHb) and nitrate (NO_3^-).

The reaction between NO and deoxygenated haemoglobin forms iron-nitrosyl haemoglobin (equation 1.5).

$$NO + Hb \longrightarrow HbNO$$

Equation 1.5: Formation of iron-nitrosyl haemoglobin (HbNO) from the reaction of NO with deoxygenated haemoglobin (Hb).

The major NO metabolite species observed in blood under normal physiological conditions are nitrate and nitrite with smaller amounts of S-nitrosothiols, N-nitroamines and HbNO (Kelm, 1999).

1.11 Functional effects of NO

In addition to its effects on vascular tone NO also exerts other functions that underlie its critical role in sustaining vascular health. The endothelial response to platelets is controlled by 3 major mediators, serotonin, ADP and thrombin, which act on 5-HT1D serotonin and P2Y purinergic receptors respectively. The endothelial action of thrombin and platelet products is crucial for the protective role played by the normal healthy endothelium against unwanted coagulation. Local platelet aggregation along with the release of serotonin, ADP and the production of thrombin (from the local activation of the coagulation cascade), leads to a significant local release of NO that diffuses into the underlying vascular SMCs inducing relaxation and dilation of the artery. This process aids elimination of the microaggregate. In addition to vasodilation NO exerts a number of effects directly on the platelet, which also attenuate platelet reactivity. Furthermore, release of NO toward the blood vessel lumen also inhibits platelet adhesion to the endothelium. Thus the tonic NO generation in a healthy blood vessel, in synergy with prostacyclin, is thought to eliminate the potential danger of vascular occlusion (Radomski *et al.*, 1990).

The direct actions of NO to inhibit platelet activation (Stamler *et al.*, 1989; Cooke *et al.*, 1990) and prevent thrombosis are thought to be mediated in part through inhibition of the normal activation-dependent increase in the expression of platelet surface glycoproteins such as P-selectin and the integrin glycoprotein IIb/IIIa complex (Michelson *et al.*, 1996). These effects of NO have been shown to be due to sGC stimulation resulting in cGMP-dependent protein kinase stimulation leading to a reduction in binding of fibrinogen to glycoprotein IIb/IIIa and modulation of phospholipase A2- and C-mediated responses (Radomski *et al.*, 1993). However, there is some controversy regarding this issue with some suggestions in sGC deficient mice that cGMP increases platelet activation (Marjanovic *et al.*, 2005; Stojanovic *et al.*,

2006; Zhang *et al.*, 2011). However, contrasting publications also in sGC knock outs (KOs) and several other studies demonstrating NO-mediated repression of platelets support NO-mediated reductions in platelet reactivity (Dangel *et al.*, 2010; Rukoyatkina *et al.*, 2011). Perhaps most relevant to this thesis are the effects of NO in inflammation. Strong evidence suggests that in health the NO released tonically by the endothelium plays a critical role in repressing inflammation within the blood vessel wall.

1.11.1 NO and its role in inflammation

The inflammatory response is the response of the host that is required to inactivate or destroy invading pathogens, remove irritants as well as preparing the site of damage for tissue repair. The response consists of specific immunological attack and non-specific immune reactions. The primary drivers in the process of inflammation include an increase in vascular permeability, release of lipid-derived autacoids or release of cytokines such as interleukin-1 (IL-1), release of small peptides such as bradykinin as well as amines such as histamine from injured tissues and migrating cells. NO has been proposed to play an influential role at almost every stage in the development of inflammation (table 1.2). In particular NO has been implicated in each step of the inflammatory cell recruitment process which is a sequential, multistep cascade that initially requires the rolling of leukocytes to endothelial cells (Rao *et al.*, 2007). The initial process is a mechanism that supports leukocyte tethering and rolling (step 1), firm adhesion (step 2) and transmigration (step 3) under laminar shear stress. Each of these steps invites interplay between soluble mediators including chemoattractants and adhesion molecules.

Symptom	Mediators
Vascular permeability	Vasoactive amines, Bradykinin, Leukotrienes (C_4, D_4, E_4), PAF, Complement (C3a and C5a), NO
Vasodilatation	NO, PGI_2, PGE_1, PGE_2, PGD_2, Hydrogen peroxide
Vasoconstriction	Thromboxane A_2, Leukotrienes (C_4, D_4, E_4), Superoxide
Chemotaxis and leukocyte adhesion	Chemokines, LTB_4, Complement (C5a), Superoxide
Pain	Bradykinin, Prostaglandins, Superoxide
Fever	IL-1, TNF, IL-6, Prostaglandins
Tissue and endothelial damage	Reactive oxygen species, Nitric oxide (iNOS), Lyzosomal enzymes

Table 1.2: Summary of the various processes in inflammation that NO might contribute to (adapted from (Guzik *et al.*, 2003)).

NO produced by the endothelium acts as an endogenous inhibitor of neutrophil adhesion to vascular endothelium. In 1991, it was first proposed that inhibition of NOS in the microvasculature would lead to an increase in leukocyte recruitment. Quantification, using intravital microscopy of NO-dependent leukocyte (mostly neutrophil) adhesion was conducted in cat mesenteric venules superfused with the NO synthase inhibitor L-NAME or L-NMMA (Kubes *et al.*, 1991). Use of either inhibitor caused a significant increase (L-NMMA=16-fold; L-NAME=21-fold) in leukocyte adherence with promotion of emigration, 2 events generally associated with microvascular dysfunction. Reversal of L-NAME-induced inhibition was possible with L-arginine. In this particular study the authors also used the CD18-specific antibody IB_4 and demonstrated attenuation of the adherence properties of the leukocyte in the presence of blockade of the adhesion glycoprotein CD11/CD18. The authors suggested that NO also targets this adhesion molecule since the effect of the NOS inhibitor L-NMMA or L-NAME were prevented in the presence of antibody (Kubes *et al.*, 1991). Furthermore pretreatment of cats with L-arginine prevented L-NAME-induced increases in microvascular protein and fluid flux as well as vascular protein clearance (Kubes *et al.*, 1992). Similar observations have also been shown in rat and mouse mesentery (Davenpeck *et al.*, 1994; Ahluwalia *et al.*, 2004)

Similarly, in rat mesentery exposure to L-NAME initially caused leakage into the interstitial space that was also followed by leukocyte adherence and emigration. In this study, as previously, attenuation of expression of the adhesion molecules CD11/18 was observed but the authors also demonstrated suppression of intercellular adhesion molecule-1 (ICAM-1) or P-selectin (CD62P) (Kurose *et al.*, 1993) an observation supported by others (Davenpeck *et al.*, 1994) but also by studies in mice. Ahluwalia et al demonstrated elevated leukocyte rolling at rest and following IL-1β in eNOS KO mice vs. WT mice: an effect that was associated with upregulated P-selectin expression at the level of the endothelial cell (Ahluwalia *et al.*, 2004). In line with this, NO donors such as nitroprusside also exhibit anti-inflammatory effects. Superfusion with nitroprusside reversed L-NAME induced vascular protein and fluid leakage in cat mesentery (Kubes *et al.*, 1992).

These effects of endogenous and exogenously delivered NO upon cell recruitment appear to be mediated by activation of cGMP. In the study of Kurose et al L-NAME induced adhesion in rat mesenteric venules was reversed with a cGMP analogue (Kurose *et al.*, 1993). Furthermore, studies in mice demonstrate that activators of sGC such as the Bayer compound BAY 41-2272 mimic the effect of endothelial-derived NO and reduce the raised leukocyte rolling evident in eNOS deficient mice (Ahluwalia *et al.*, 2004).

The inhibitory action of NO on leukocytes has been shown to extend to monocytes as well as neutrophils. The interaction of endothelial cells with circulating monocytes is a key part of the normal host-defense pathways in normal vascular biology. Indeed, the constant on/off interaction of monocytes with the endothelium has been described as a mechanism of survival and the particular subset of monocytes that do this are termed ‘patrolling’ monocytes in some instances (Shi *et al.*, 2011). However, prolonged interactions between these cells and the endothelium are implicated in the pathogenesis of vascular disease. Monocyte adhesion to porcine aortic endothelial cells was shown to increase in response to the chemoattractant N-formyl-methionyl-

leucyl-phenylalanine (fMLP). However, bubbling of pure NO into the bathing medium resulted in a reduction of adhesion. Since no change in the leukocyte adhesion molecule CD11b/18 was evident in these experiments it was suggested that the effects were related to changes at the level of the endothelium rather than on the monocyte itself. In the presence of superoxide dismutase (SOD) the effects of NO were enhanced and this was associated with increased levels of endothelial cGMP, demonstrating that prevention of NO scavenging by superoxide lead to repression of monocyte adhesion by influencing the endothelium (Bath *et al.*, 1991). In another study IL-1α-stimulated human saphenous vein endothelial cells were used to investigate NO-mediated monocyte adhesion. It was shown that 3 structurally unrelated NO donors (SNP, SIN-1 and GSNO) inhibited VCAM-1 expression (De Caterina *et al.*, 1995). For maximal inhibition of adhesion molecule expression the presence of the NO donors throughout the period of stimulation was required. This reduction in VCAM-1 expression was paralleled by reduced monocyte adhesion to endothelial monolayers. Furthermore inhibition of endogenous NO production induced VCAM-1 expression (De Caterina *et al.*, 1995). Finally, it has been suggested that at least part of the suppression of monocyte adhesion relates to a suppression of chemokine expression. In human studies a specific suppression of monocyte chemoattractant protein-1 (MCP-1) has been demonstrated (Desai *et al.*, 2006). Thus, endothelial-derived NO prevents leukocyte adhesion and their subsequent migration into the subendothelial space. The anti-leukocyte properties of NO are mediated through inhibition of the expression of endothelial cell surface adhesion molecules P-selectin, vascular adhesion molecule-1 (VCAM-1), ICAM-1 as well as repression of leukocyte CD11/18 and prevention of the expression of monocyte chemoattractant protein-1 (MCP-1).

Interestingly, it has been suggested that during vascular adherence a number of phenomena occur that influence endothelial NO-induced repression of cell recruitment. The adhesion of monocytes to the endothelium itself was shown to suppress the release of biologically active NO in a monocyte concentration- and time-dependent manner (Marczin *et al.*, 1996). Investigations of the effects of monocytes on the NO pathway on cultured endothelial cells have revealed that the attenuation of

NO release is also due to reduced levels of eNOS. It was suggested that such an effect would naturally predispose a vessel to form atherosclerotic lesions due to a combination of effects such as thrombosis and increased recruitment of platelets and leukocytes to the site of inflammation (Marczin *et al.*, 1996).

Thus it is clear that NO plays an important role in keeping the blood vessel in an anti-inflammatory state that is repressive for inflammatory cell recruitment. In CVD the presence of endothelial dysfunction that is characterized by reduced bioavailability of endothelial NO thus places the blood vessel vulnerable to inflammatory cell recruitment.

1.12 The key events in endothelial dysfunction

The term 'endothelial dysfunction' stems from an observation that Ludmer and co-workers made in 1986 when they reported that ACh-induced vasorelaxation was impaired, even reversed, in atherosclerotic coronary arteries in humans (Ludmer *et al.*, 1986). The investigators observed paradoxical constriction in the arteries of patients with CAD as well as in those with advanced CAD, indicating that endothelial dysfunction is present in the early stages of atherosclerosis (Ludmer *et al.*, 1986). In addition in studies looking at either ACh-induced vasorelaxation or measurement of flow mediated dilatation (FMD), endothelial dysfunction was detected in both the conduit and microvascular blood vessels in patients with coronary risk factors (Celermajer *et al.*, 1994; Reddy *et al.*, 1994). Brachial artery ultrasound measurement of FMD is commonly used to assess endothelial function in humans and is the gold standard for demonstrating endothelial dysfunction in CVD patients (Corretti *et al.*, 2002).

Atherosclerosis, and diseases which predispose to atherosclerosis such as hypercholesterolaemia, diabetes and hypertension, are characterized by endothelial dysfunction (Cai *et al.*, 2000). Under this particular state endothelial cell function is compromised leading to altered activity of platelets, leukocytes and the underlying vascular SMCs. Experimentally endothelial dysfunction is commonly characterized by

demonstrations of defects in the normal vaso-relaxation response to mediators such as ACh and/or to increased blood flow. The underlying basis for endothelial dysfunction is thought to involve a reduction in bioavailable NO in the vasculature. There are several mechanisms that are thought to underlie the reduced bioavailability in NO and these can broadly be divided into 3 categories: reduced expression of eNOS, reduced eNOS enzymatic activity and rapid removal of NO.

Expression of eNOS may be reduced at the vessel wall due to reduced transcription and/or increased instability of eNOS mRNA caused by cytokines (Oemar *et al.*, 1998). Indeed there are numerous demonstrations of reduced eNOS expression in models of CVD, including atherosclerosis (Oemar *et al.*, 1998; Won *et al.*, 2007) and hypertension (Giaid *et al.*, 1995; Chou *et al.*, 1998). However, this issue is controversial since in several animal models of atherosclerosis an unchanged or even augmented expression of eNOS, at least in early atherosclerosis, despite the presence of impaired endothelium-dependent relaxations has been demonstrated (Kanazawa *et al.*, 1996; d'Uscio *et al.*, 2001).

Under pathological conditions it is now well accepted that eNOS can switch from an NO-producing enzyme to an enzyme that generates ROS. This phenomenon is termed eNOS uncoupling (see figure 1.4) and results in superoxide formation if the key cofactor BH_4 is not present (Alp *et al.*, 2004), or generation of hydrogen peroxide if the substrate L-arginine is deficient (Vasquez-Vivar *et al.*, 1998; Cai *et al.*, 2005; Crabtree *et al.*, 2011). Laursen et al demonstrated the production of superoxide from eNOS. In apolipoprotein E knock out (ApoE KO) mice the authors showed the increased vascular superoxide production from the endothelium was associated with impaired endothelial function. Incubation of vessels with sepiapterin (a precursor to BH_4) decreased superoxide formation and improved endothelial function (Laursen *et al.*, 2001). The enzymatic activity of eNOS is inhibited by various mechanisms associated with atherosclerosis and hyperlipidemia. Pro-atherogenic lipids, such as oxidized low-density lipoprotein (oxLDL) inhibit signal transduction from receptor activation to eNOS activation (Hirata *et al.*, 1991). Hypercholesterolemia and LDL upregulate

caveolin abundance, augment the caveolin-eNOS complex and therefore attenuate NO production from the endothelial cell (Feron *et al.*, 1999; Feron *et al.*, 2001). Endogenous NOS inhibitors e.g. asymmetric dimethylarginine (ADMA) and N-monomethylarginine (NMA) are also thought to contribute to a reduction in the endothelium-dependent relaxation response in CVD (Miyazaki *et al.*, 1999; Cooke, 2000) since levels of these are elevated in both atherosclerosis (Boger *et al.*, 1997) and hypertension (Surdacki *et al.*, 1999).

Increased superoxide production causing accelerated NO degradation is another key mechanism in endothelial dysfunction (Harrison, 1997). In animal models of atherosclerosis and in patients there is evidence of an increase in superoxide production (Ohara *et al.*, 1993; Rikitake *et al.*, 2001). The endothelium is an important source of superoxide production and its denudation decreases superoxide production from vessels that have atherosclerosis compared to no effect being observed in normal vessels that do not have atherosclerosis (Ohara *et al.*, 1993). As well as uncoupled eNOS additional important endothelial sources of superoxide in pathological conditions include XOR (Berry *et al.*, 2004) and NADPH oxidase (Frey *et al.*, 2009; Manea, 2010). In vivo models of hyperlipidemia and atherosclerosis are associated with excessive amounts of superoxide that is linked to reduced bioavailable NO. Excessive superoxide production has been confirmed by the use of antioxidants and superoxide dismutase in restoring impaired endothelium responses in atherosclerotic vessels (Mugge *et al.*, 1991; Drexler, 1997). In rabbit aortae with high-cholesterol diet-induced atherosclerosis, the impaired vasodilatory responses to ACh and A23187 (calcium ionophore) were restored by chronic treatment with polyethylene-glycolated SOD (Mugge *et al.*, 1991). Furthermore antioxidants improve endothelial function in human and animal models with atherosclerosis (Taddei *et al.*, 1998; Beckman *et al.*, 2001) in particular vitamin C is effective in the restoration of endothelium dysfunction from risk factors such as hypercholesterolemia, hypertension and diabetes mellitus (Levine *et al.*, 1996; Ting *et al.*, 1996; Ting *et al.*, 1997).

Endothelial dysfunction was first described in human hypertension in the forearm vasculature in 1990 (Panza *et al.*, 1990). Impaired vasodilation in hypertension has been confirmed by many studies in different vascular beds, including small resistance vessels (Schiffrin *et al.*, 2000; Park *et al.*, 2001). Although human and animal studies have demonstrated that hypertension is accompanied by an increase in oxidative stress, the evidence for this in humans is not definitive (Oparil *et al.*, 2003). Clinical studies have shown that there is an increase in production of oxygen-derived free radicals (Mehta *et al.*, 1994), together with a decreased level of antioxidants (Sagar *et al.*, 1992). Although studies in humans have not been as convincing as those in experimental models the increase in oxidative stress has been shown in various types of hypertension such as essential hypertension, renovascular hypertension, salt-sensitive hypertension and preeclampsia (Higashi *et al.*, 2002; Lee *et al.*, 2003; Fortuno *et al.*, 2004). Furthermore, plasma levels of ADMA and 13-hydroxyoctadecadienoic acid (a marker of ROS production) are both elevated in patients with hypertension, and microvessels from patients with the highest levels of these markers are least responsive in terms of NO-induced relaxation (Wang *et al.*, 2009).

Thus NO is a critical molecule in cardiovascular physiology. Release of NO from the endothelium regulates vascular homeostasis, however numerous CVDs or risk factors impair NO synthesis or increase NO destruction. The overall effect is the enhancement of vascular tone and reactivity as well as predisposition to atheroma formation. Thus development of novel strategies to replenish reduced NO levels have clear therapeutic potential. The identification of endogenous NO generating pathways independent of the conventional L-arginine/NOS pathway centres on the chemical reduction of nitrite and have recently offered new opportunities to deliver NO in CVD.

1.13 Introduction to nitrite in physiology

In 1953 Furchgott demonstrated that nitrite relaxed spirally cut strips of rabbit thoracic aorta, however this only occurred at high concentrations of nitrite (100 and 1000µM). Low concentrations were not actually tested therefore it was inferred that at least at physiological levels nitrite had no biological activity (Furchgott *et al.*, 1953).

Thus in 2001 nitrite was generally viewed as having little physiological function and was largely viewed as a useful marker of endogenous NO production and reflective of NOS activity. Indeed studies in healthy volunteers confirm that nitrite rather than nitrate provides an accurate estimate of eNOS activity (Lauer *et al.*, 2001). In blood collected from 24 healthy volunteers from the antecubital vein and the brachial artery at rest there was no difference in nitrite or nitrate levels between the arterial and venous circulations (Lauer *et al.*, 2001). However, following infusion of the eNOS inhibitor L-NMMA dose-dependent decreases in blood flow were evident and associated with dose-dependent decreases in nitrite but no changes in nitrate. Furthermore stimulation of eNOS with ACh augmented forearm blood flow almost 4-fold and the change was paralleled with increases in plasma nitrite concentration and no significant changes in plasma nitrate concentration. In this study the authors also infused nitrite into the forearm at doses of 0.01-36μmol/min, levels that resulted in elevations of venous nitrite, however no change in forearm blood flow was evident. Importantly the authors measured venous plasma nitrite concentrations, which exceeded 130μM and found that they were approximately 200 times greater than the concentrations measured during maximal eNOS stimulation with ACh. The results demonstrated that circulating levels of nitrite are a good reflection of eNOS activity but that nitrite per se was not responsible for change in blood flow and possessed no vasodilator activity, essentially confirming the view that Furchgott's studies in 1953 had established (Lauer *et al.*, 2001). This view contradicted proposals that nitrite might play a role in NO delivery, however in 2003 this view was strongly challenged by studies demonstrating exactly the contrary.

In 2000, work published by Gladwin and colleagues showed significant arterial-venous gradients for plasma nitrite demonstrating that at baseline arterial nitrite levels (540±74nM) were significantly greater than venous nitrite levels (466±79nM) (Gladwin *et al.*, 2000). Even though this work demonstrated the existence of the arterial-venous gradient, suggesting that nitrite is reduced to NO under normal physiological

conditions, others supported the view that the gradient was actually a result of differences in NOS activity (Luchsinger *et al.*, 2005).

In 2001, using rat aorta in vitro, the vasorelaxant effects of physiological levels of nitrite was demonstrated where in phenylephrine precontracted aortic segments sodium nitrite caused dose-dependent relaxation (Modin *et al.*, 2001). In a neutral buffer (pH 7.45) the threshold dose was 10µM, the EC50 was 200µM and the aortic segment relaxed to almost basal tone following 1000µM nitrite, which was the highest concentration tested. In acidic buffer (pH 6.66) the threshold dose was 2.5µM and the EC50 was 40µM, therefore a leftward shift of the concentration-response curve was observed. The nitrite-derived relaxant effects were blocked by the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ). The results showed that nitrite is a more potent vasodilator in acidic conditions and its vasorelaxant effects were mediated through NO generation (Modin *et al.*, 2001).

The ability of nitrite to act as a vasodilator was further elucidated when it was shown that nitrite infusion into the human forearm brachial artery resulted in increased forearm blood flow (Cosby *et al.*, 2003a). Forearm blood flow measurements were made in 18 healthy volunteers and nitrite infused at 36µmol/ml, into the brachial artery to achieve an intravascular nitrite concentration of approximately 200µM (Cosby *et al.*, 2003a). Systemic nitrite concentration of 16µM, measured in the contralateral arm, was associated with a systemic decrease in mean arterial blood pressure of approximately 7mmHg. The physiological relevance of vascular nitrite as a vasodilator was tested. An infusion of 400nmol/ml significantly increased forearm blood flow in all subjects tested. Blood flow was significantly increased at rest and during NOS inhibition, with and without exercise to simulate hypoxia. Following a 5 minute infusion of nitrite, venous nitrite levels initially increased from 176±17nM to 2,564±462nM. Following exercise venous nitrite levels decreased to 909±113nM (Cosby *et al.*, 2003a). These observations lead to the acceptance that nitrite is functional and could act as a storage pool for NO.

Coupled with the observation that arteriovenous gradients exist in the human forearm was the knowledge that these gradients were enhanced during exercise and regional NOS inhibition. This lead to the suggestion that nitrite is consumed to liberate NO (Gladwin *et al.*, 2000; Cosby *et al.*, 2003a). Still, around that time the involvement of nitrite at physiological concentrations on vasodilation was disputed. It was thought that the nitrite gradient may be due to decreased production of nitrite on the venous side through a combination of decreased NOS activity and increased nitrite consumption (Luchsinger *et al.*, 2005). Maher and colleagues therefore set out to determine whether nitrite would have a greater effect in capacitance vessels compared to resistance vessels due to lower oxygen tension. Furthermore, whether resistance-vessel dilation would be more pronounced during hypoxemia. The effect of intra-arterial infusion of nitrite on forearm blood flow was assessed during normoxia and hypoxia. There was no significant difference between dilation in the capacitance vessels under normoxia compared with hypoxia (35.8$\pm$3.4% for normoxia). In the resistance vessels nitrite-induced vasodilation, as assessed by the forearm blood flow ratio, was moderately increased from baseline (1.04$\pm$0.09 to 1.62$\pm$0.18FBF-R, $p<0.05$). In hypoxia, however, arterial blood flow was increased over 2 fold from baseline (1.07$\pm$0.09 to 2.37$\pm$0.15, $p<0.005$). In addition, in vitro studies using vessels isolated from rabbits confirmed the in vivo observations giving credence to the suggestion that hypoxia significantly influences nitrite-induced relaxation in both arteries and veins (Maher *et al.*, 2008).

1.14 Conversion of nitrite to NO

In 1994 2 groups independently and for the first time demonstrated that nitrite can be reduced to NO in a biological system (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994). In these studies NO production was measured in expelled air of healthy volunteers using the highly specific ozone chemiluminescence assay. At the time it was known that human saliva contained both nitrate and nitrite and that nitrate absorbed in the gastrointestinal tract is extracted by the salivary glands from plasma (Spiegelhalder *et al.*, 1976; Tannenbaum *et al.*, 1976). Furthermore most of the nitrite in the saliva resulted from the reduction of nitrate within the oral cavity, a process facilitated by

micro-organisms residing normally within the oral cavity (Tannenbaum *et al.*, 1976). It was logical to postulate that swallowed saliva would enter the stomach and under the acidic environment presented by the stomach reduction of nitrite to NO may occur (Archer, 1993). Lundberg et al. demonstrated that basal NO concentration in expelled air after 10 hours of fasting was approximately 602ppb in healthy volunteers, however following ingestion of a dietary nitrate load (in the form of lettuce which is naturally rich in nitrate) exhaled NO levels were increased 4 fold, 5 minutes following the intake of lettuce. In vitro they demonstrated that NO formation from nitrite was pH dependent between pH 1 to 2 (Lundberg *et al.*, 1994). The requirement of an acidic environment for NO formation from nitrite was confirmed by treating volunteers with the selective proton pump inhibitor omeprazole. Omeprazole inhibits gastric acid secretion and use of this inhibitor almost abolished NO in expelled air (Lundberg *et al.*, 1994). At these particularly high levels NO is toxic to a wide range of microorganisms and therefore this pathway may reflect a mechanism of host defense. Indeed Benjamin and colleagues investigated this hypothesis by exposing enteropathogens to different combinations of acid and nitrite (Benjamin *et al.*, 1994). They proposed that acidification of nitrite in the stomach would result in the formation of nitrous acid followed by spontaneous decomposition to NO and other nitrogen oxides (see equations 1.6-1.8). Thus simple nitrite diproportionation, which increases under acidic conditions with abundant availability of H^+ ions, favours the release of free NO.

Equation 1.6: $NO_2^- + H^+ \longleftrightarrow HNO_2$

Equation 1.7: $2HNO_2 \longleftrightarrow H_2O + N_2O_3$

Equation 1.8: $N_2O_3 \longleftrightarrow NO\cdot + NO_2\cdot$

Equations 1.6, 1.7 and 1.8: Equations depicting conversion of nitrite to nitric oxide (NO) in the stomach. In the acidic environment of the stomach, nitrite (NO_2^-) exists in equilibrium with nitrous acid (HNO_2) (equation 1.6). Both exist in equilibrium with oxygen intermediates including dinitrogen trioxide (N_2O_3) (equation 1.7), which breaks down to form NO and nitrogen dioxide (NO_2) (equation 1.8).

Very soon after these studies were published Zweier and colleagues demonstrated that nitrite reduction also occurs within the cardiovascular system in acidotic conditions (Zweier *et al.*, 1995). The authors observed that in ischaemic tissues such as the heart, large quantities of NO, measured by EPR spectroscopy were produced via a pathway that was independent of the conventional L-arginine/NOS pathway. Using the ferrous iron complex of N-methyl—D-glucamine-dithiocarbamate (MGD), Fe(II)-MGD2 (Fe-MGD) to trap NO rat hearts perfused at a pressure of 80mmHg infused with Fe-MGD for 5 minutes and then subjected to 30 minutes global ischaemia (Zweier *et al.*, 1995). They demonstrated that inhibition of NOS reduced NO formation during the ischaemia by more than 60% although it was never fully blocked. However, this group demonstrated that fairly high levels of nitrite were present in the heart (20µM). They then investigated the possibility that this nitrite might be the source of NO since during ischaemia they showed that pH decreased down to pH 5.5 and hypothesized that this acidosis might be sufficient to chemically reduce nitrite to NO. Using ^{15}N nitrite they demonstrated that during ischaemia nitrite could be directly reduced to NO and thus postulated that nitrite sequestered in the cardiac tissue was responsible for a considerable proportion of NO generation during the ischaemia as a consequence of acidic disproportionation (Zweier *et al.*, 1995).

1.15 Mechanism of nitrite conversion to NO

As demonstrated, in an acidic environment simple chemical disproportionation encourages the release of free NO. In tissues, however, the process of chemical disproportionation accounts for only up to 20% of the total NO produced from nitrite and we now know that the rest is derived from enzymatic nitrite reduction. This view was first demonstrated in cardiac tissue where nitrite reduction facilitated by rat heart homogenates was reduced by ~75% after proteins were denatured (Webb *et al.*, 2004a). In this study it was shown that this dependency on protein relates to enzymatic activity that could be attributed to xanthine oxidoreductase (XOR). Since that time a number of mammalian nitrite reductases have been identified (figure 1.5) that can be broadly grouped into 3 categories. These are (1) haem based nitrite reductases e.g. haemoglobin (Huang *et al.*, 2005), myoglobin (Hendgen-Cotta *et al.*,

2008) and neuroglobin (Tiso *et al.*, 2011), (2) the nitrite anhydrases e.g. carbonic anhydrase (Aamand *et al.*, 2009), and (3) the molybdopterin reductases e.g. aldehyde oxidase (Li *et al.*, 2009) and XOR (Millar *et al.*, 1998a; Li *et al.*, 2001b). In the work described in this thesis the focus has rested on the third group and the next section of this chapter describes in detail this activity.

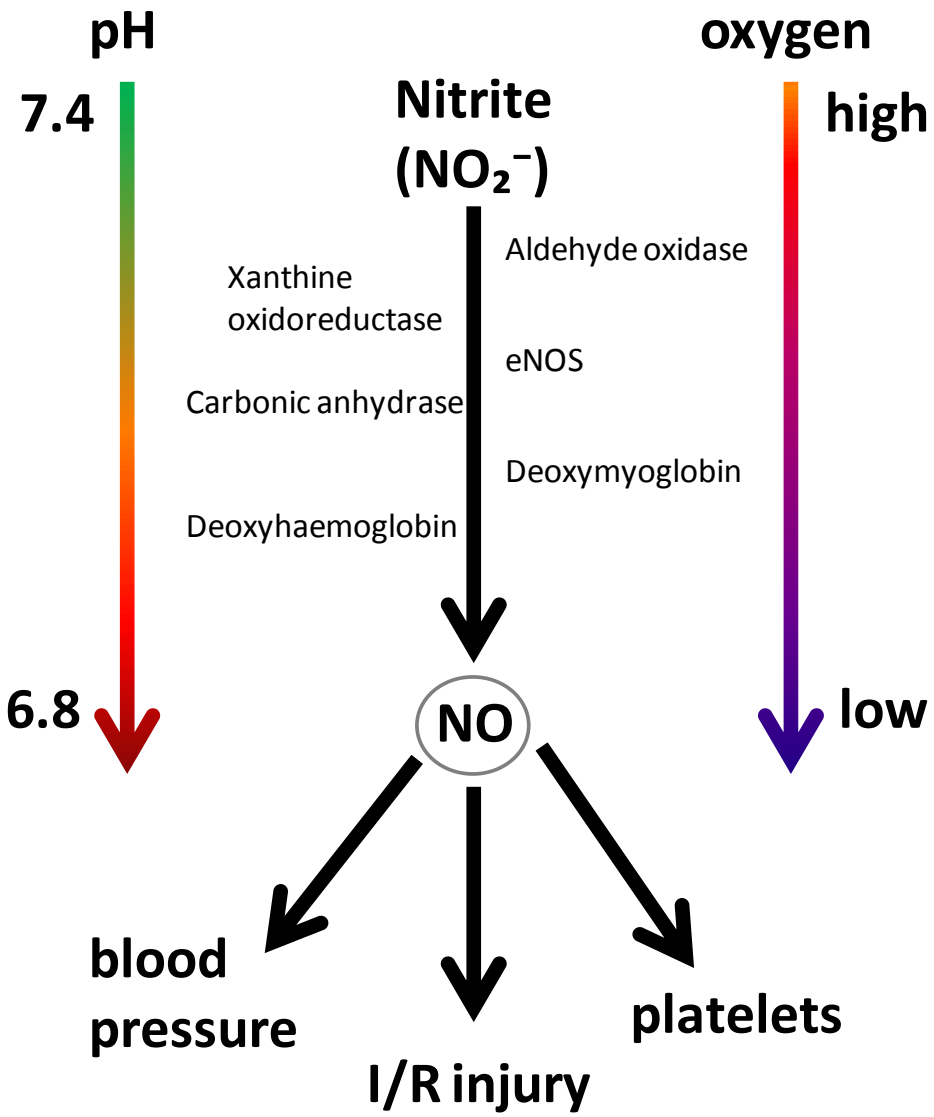


Figure 1.5: The enzyme-mediated pathways for nitrite reduction. Conditions such as hypoxia and acidosis enhance the magnitude of nitrite reduction to NO, a process facilitated by numerous nitrite reductases.

1.15.1 Enzymatic conversion by the molybdopterin reductases

The family of molybdenum (Mo) containing reductase enzymes catalyse a wide range of reactions. These reactions are dependent on the oxidation states of Mo, which can

exist in a range of oxidation states from +4 to +6 (Mo(IV), Mo(V), Mo(VI) (Zhang *et al.*, 1998a). There are over 50 different Mo-containing enzymes, the vast majority of which are bacterial enzymes (Hille, 2013), however in mammals there are 4 known Mo-containing enzymes: sulphite oxidase (SO), aldehyde oxidoreductase (AO), mitochondrial amidoxime reducing component (mARC) and XOR (figure 1.6). Interestingly, there is now evidence that all of these Mo-containing enzymes can act as nitrite reductases (Stirpe *et al.*, 1969; Sparacino-Watkins *et al.*, 2013; Wang *et al.*, 2013). However, the most well described and characterized of these enzymes is XOR.

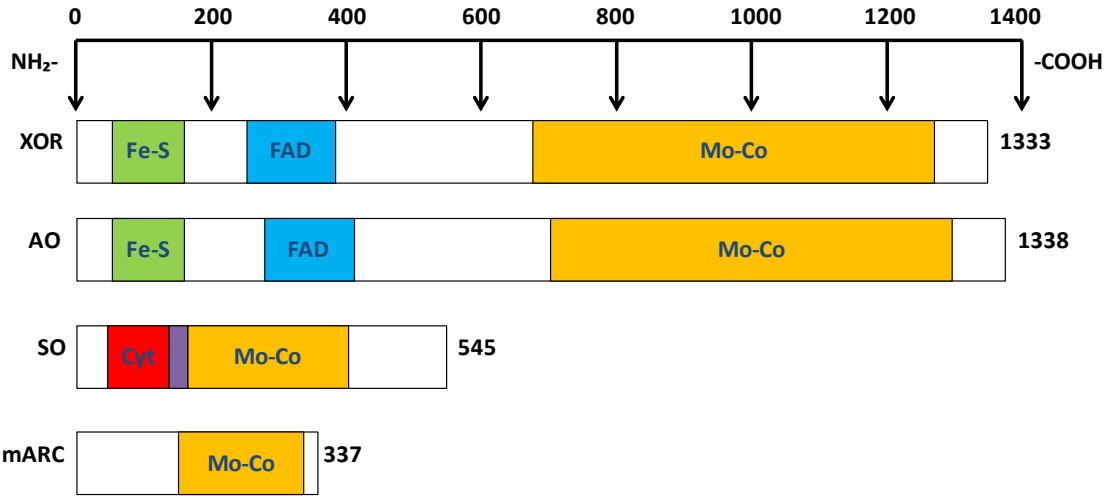


Figure 1.6: Secondary structure of the 4 known mammalian molybdenum containing enzymes XOR, AO, SO and mARC. AO=aldehyde oxidase; SO=sulphite oxidase; mARC=mitochondrial amidoxime reducing component.

1.15.2 Introduction to xanthine oxidoreductase (XOR)

In 1902 the Austrian biochemist Franz Schardinger found an enzyme fraction in whole milk decolourizes methlyene blue in the presence of formaldehyde (Schardinger, 1902). After this discovery, some 20 years later, the same enzyme fraction was found to produce urate (Morgan *et al.*, 1922). Urate production was then shown to belong to the terminal rate-limiting steps in human purine catabolism from the degradation products hypoxanthine and xanthine by the same Schardinger enzyme, now known to be XOR.

XOR refers to 2 interconvertible forms found in mammalian cells, xanthine oxidase (XO) and xanthine dehydrogenase (XDH) (Enroth *et al.*, 2000). XOR exists as a

homodimer (Andrews *et al.*, 1964) with a single monomer having a molecular weight of approximately 150kDa. Each monomer comprises a flavin adenine dinucleotide (FAD) site, 2 iron-sulphur (2Fe-2S) clusters (Richert *et al.*, 1954) (residing at the N-terminus of each monomer) and a molybdopterin (Mo) site that resides at the C-terminus.

Transcription of the gene encoding XOR results in the expression of XDH, which under physiological conditions is the predominant intracellular form whereas XO is a post-translationally modified product of the initial XDH form. XDH can be reversibly converted to XO via sulphydryl oxidation or irreversibly due to proteolysis (Della Corte *et al.*, 1968). Both forms of XOR catalyze the hydroxylation of purines at the Mo binding site but the difference between the enzymes lies in the activity of the flavin cofactor. The catabolism of purines occurs at the FAD site but for XDH the electron acceptor is NAD^+ (although it can use molecular oxygen as well) whilst for XO the electron acceptor is only molecular oxygen (Hille *et al.*, 1995; Enroth *et al.*, 2000; Okamoto *et al.*, 2013).

Electron flow begins at the Mo site from a substrate that is either a purine or an aldehyde. The resultant electron flow changes the Mo oxidation state from +6 to +4 (Stockert *et al.*, 2002). The 2 electrons are distributed sequentially to the nearest Fe-S cluster positioned 10-15Å away. Following transfer to the next Fe-S cluster (Eger *et al.*, 2000) the electrons are passed onto the FAD site (located 10-12Å away from the Fe-S cluster) resulting in the formation of FADH and then $FADH_2$ prior to reduction of the final exogenous oxidant. This process of electron transfer occurs independently in each monomer (Massey *et al.*, 1969). Depending on the form of XOR, electrons are either transferred to NAD^+ or molecular oxygen (figure 1.6). As XO only uses molecular oxygen as its terminal electron acceptor substantial superoxide generation occurs when this form of XOR is active (Hille *et al.*, 1981). The hypoxic environment prevalent in I/R injury causes XDH to preferentially utilize NADH as substrate to generate electrons. Upon reperfusion molecular oxygen uses the electrons to make superoxide and hydrogen peroxide. Studies with XOR in endothelial cells show that superoxide

generation as a consequence of reperfusion is not blocked by the Mo site targeted inhibitor allopurinol but by the FAD site targeted inhibitor diphenyleneiodonium (Harris *et al.*, 1997).

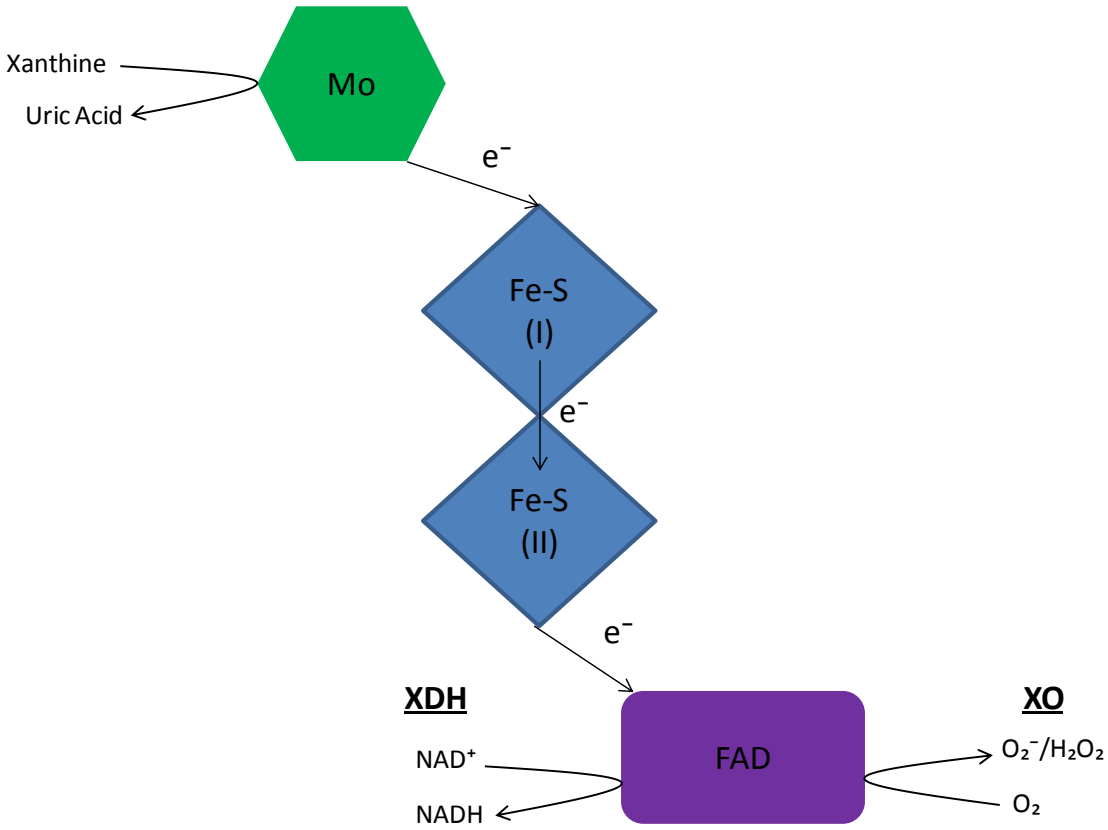


Figure 1.7: Electron flux through 1 monomer of XOR. Arrows show the direction of electron flow. Depending on the form of XOR the reduced FAD site reacts with either NAD^+ or molecular oxygen to produce NADH or hydrogen peroxide (H_2O_2) or superoxide (O_2^-). $FADH_2$ reacts with O_2 to produce H_2O_2, while FADH produces O_2^-.

1.15.3 How does XOR function as a nitrite reductase?

Under anaerobic conditions and in the presence of either NADH or xanthine, purified XOR can also catalyse the reduction of nitrite to NO (Godber *et al.*, 2000; Li *et al.*, 2001a). The process of nitrite reduction was first described to occur under hypoxic conditions and as a process functioning optimally in the presence of NADH as the reducing substrate (Millar *et al.*, 1998b; Millar *et al.*, 1998a; Zhang *et al.*, 1998b). Interestingly, it has been known for almost 90 years that XOR can catalyze the reduction of nitrate to nitrite (Dixon *et al.*, 1924). In these early studies it was not

considered whether nitrite might also be reduced by the enzyme and neither what might be the biological significance of this activity. Indeed, these observations were described by the authors as simply identifying another oxidizing agent that might be used to study 'Schardinger' enzyme.

Two groups in 1998 published observations with purified enzyme demonstrating the nitrite reductase activity of XOR. Using a chemiluminescence NO detector to measure NO production, Millar and co-workers (Millar *et al.*, 1998a) demonstrated that under <1% oxygen conditions, purified bovine XOR catalyzed NO production from nitrite (using mM concentrations of nitrite), a phenomenon abolished with the introduction of oxygen. However, it is notable that this reduction of nitrite did not occur if NADH was substituted with xanthine or hypoxanthine. Indeed, if either of the purines were introduced into the reaction mixture concomitantly with NADH, nitrite reduction was inhibited, although the authors provided no explanation for why this might be the case. Zhang et al also published their observations in 1998 using bovine XOR (Zhang *et al.*, 1998b). However, unlike Millar et al they suggested that the capacity of XOR to generate nitrite for NO was not altered by the presence of oxygen (ambient air). In addition, this group demonstrated that NO production from nitrite was completely blocked by the Mo-directed inhibitor, allopurinol, or the FAD-directed inhibitor, DPI. They concluded that nitrite is likely to interact with the Mo centre, whilst NADH interacts with the FAD site. Essentially the mechanism of this reduction is the reverse of xanthine oxidation but with NADH as the reducing substrate. NADH donates electrons to FAD to form $FADH_2$. $FADH_2$ then reduces Mo, which goes from oxidation state +6 to +4. This reduced Mo then accepts O^- from nitrite to give Mo(V) and consequently releases NO. The Mo returns to its original oxidation state by obtaining an electron from the $FADH_2$ centre restoring FAD for the next cycle of nitrite reduction. Importantly, Zhang and co-workers demonstrated that this profile of activity of XOR was also shown to hold true for XOR in homogenates of inflamed human synovium. Interestingly, this group had also previously published observations using human XOR purified from human breast milk (Zhang *et al.*, 1997). In that particular study they demonstrated that allopurinol had no effect on the nitrite reductase activity of the

enzyme (Zhang *et al.*, 1997). Exactly why this difference occurred between the studies is uncertain, however, in the study with human XOR a concentration of allopurinol of only 10µM was used as opposed to mM concentrations of nitrite. Allopurinol is a reversible inhibitor of the enzyme with an IC_{50} reported to lie between 0.2 to 50µM (for review see (Pacher *et al.*, 2006)). A simple explanation for this observation is that insufficient amounts of allopurinol were used. Further extensive analysis of the nitrite reductase activity of bovine XOR was then conducted by Jay Zweier and his group (Li *et al.*, 2001a; Li *et al.*, 2004a). Collectively, the studies demonstrated that xanthine, aldehyde (in the form of 2,3-dithdroxybenz-aldehyde) and NADH all act as electron donors for nitrite reduction, but importantly with xanthine having the highest Km (See Table 1.3) (Li *et al.*, 2001a). Additionally, whilst oxypurinol (the active form of allopurinol) inhibited nitrite reduction with all three reducing substrates, DPI only inhibited the response with NADH. Furthermore, in agreement with Millar et al (Millar *et al.*, 1998b), these studies show that high concentrations of the purines attenuate nitrite reduction and the authors suggest that this inhibition likely relates to binding of the molybdenum in the reduced form preventing access to nitrite. Finally this group also demonstrate the exquisite sensitivity of the nitrite reductase activity of XOR to oxygen, whereby xanthine or aldehyde (substrates that provide electrons at the Mo site) catalysed nitrite production competes with oxygen whilst NADH-driven nitrite reduction still occurs under aerobic conditions with ~70% of the activity evident in anoxia (Li *et al.*, 2004a).

Electron Donor	**Target Site**	**Condition**	**Km**
Xanthine	Molybdenum	Anoxia	1.5µM
Aldehyde	Molybdenum	Anoxia	35.0µM
NADH	FAD	Anoxia	0.9mM
Xanthine	Molybdenum	Air (21% O_2)	58.6mM
Aldehyde	Molybdenum	Air (21% O_2)	48.5mM
NADH	FAD	Air (21% O_2)	0.2nmols^{-1}mg^{-1} (does not follow Michaelis-Menten kinetics)

Table 1.3: Effect of electron donors on Km values for purified XO-mediated NO generation from nitrite (Li *et al.*, 2001b; Li *et al.*, 2003).

In addition to substrate availability there are a number of environmental factors that also alter XOR's ability to function as a nitrite reductase. Of particular relevance to ischaemic CVD, the activity of XOR is both increased with increasing hypoxia and by increasing acidosis. The latter has been shown to occur with purified enzyme but also with tissue homogenates. The relevance of this observation is better appreciated when one remembers that hypoxia is associated with increasing acidosis. Indeed it has been demonstrated in isolated rat heart preparations subjected to a simulated heart attack, that within 20 mins of ischaemia pH dropped from 7.4 to 5.5 (Zweier *et al.*, 1999). Zweier and colleagues (Li *et al.*, 2001a) demonstrated that irrespective of substrate, and using an excess of nitrite (1mM), the rate of NO generation was enhanced with decreasing pH. Interestingly, in this study the impact of pH upon nitrite reduction was assessed in the presence of different reducing substrates, showing that whilst a decrease in pH from 7.4 to 6 caused a small increase with xanthine (i.e. 1.1 fold) that the rate of NO generation was increased 2.3 and 2.6-fold respectively with NADH and aldehyde respectively. In homogenates of both rat and human heart tissue work in my lab has shown that decreasing pH (6.0-5.0) results in a pronounced increase in NO generation, although which reducing substrate might be involved in such responses was not assessed (Webb *et al.*, 2004b). However, as oxygen tension decreases the levels of NADH will increase as mitochondrial oxidative phosphorylation is impaired, suggesting that NADH is likely to play a major role under such conditions. The mechanism of action of XOR-facilitated nitrite reduction to NO is depicted in figure 1.8.

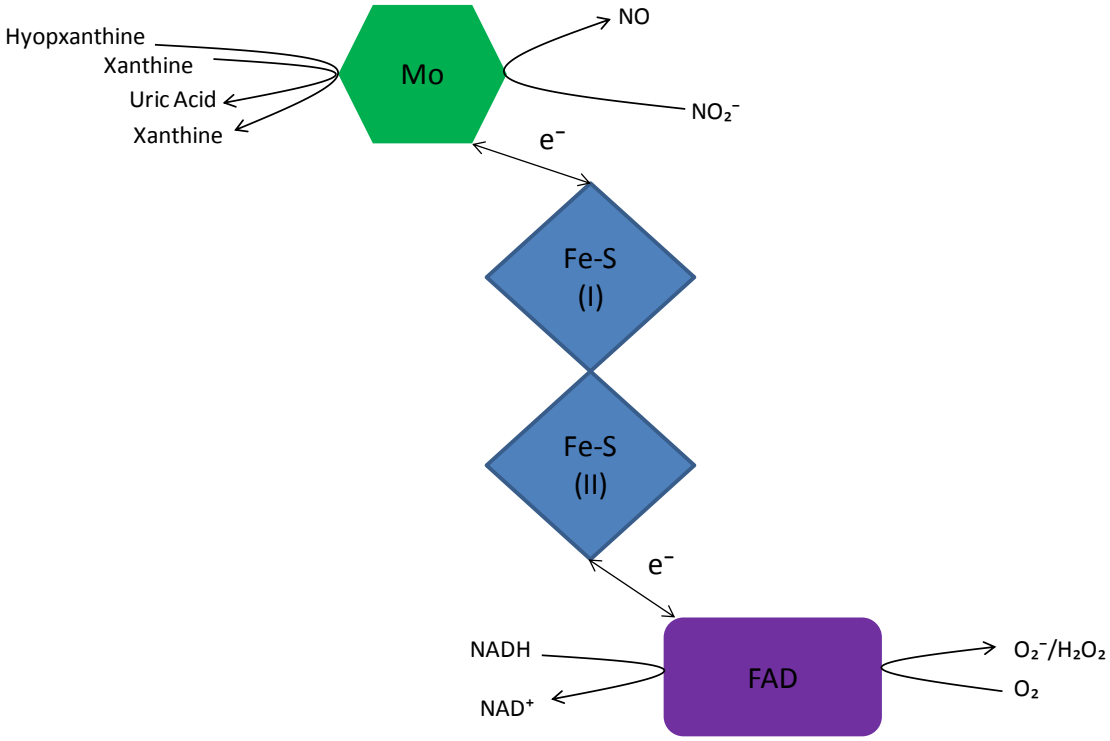


Figure 1.8: The biochemistry of XOR-facilitated nitrite reduction to NO.

1.15.4 XOR expression and distribution within the cardiovascular system

XOR is thought to be widely distributed in mammals with evidence of expression in a range of organs, however the highest levels of expression and activity appear to occur within the liver, including in humans (Linder *et al.*, 1999). Within the cardiovascular system XOR expression and activity have been identified in human heart (Abadeh *et al.*, 1992; Abadeh *et al.*, 1993). In addition, previous work in my lab has demonstrated significant nitrite reductase activity that is abolished in the presence of allopurinol in human atrial tissue, implicating XOR (Webb *et al.*, 2004b). XOR is also thought to be expressed throughout the vasculature at the level of the endothelial cell. However, despite evidence of ubiquitous capillary endothelial expression in early studies in bovine tissues (Jarasch *et al.*, 1981), in humans a more selective distribution of expression appears to be present. Early studies suggested that endothelial XOR could be found only within capillaries of certain organs including the liver, mesentery and mammary gland (Jarasch *et al.*, 1981) with little to no expression in the heart, lung, kidneys or on the endothelium of larger blood vessels of any organs. In contrast, other studies suggest expression and activity in isolated human capillary endothelial cells of

both skeletal and cardiac tissue (Jarasch *et al.*, 1986), and in addition more recent evidence suggests XOR expression in venular (human umbilical vein) endothelial cells (Kelley *et al.*, 2006; Webb *et al.*, 2008b), and in endothelial cells of small arteries (Hellsten-Westing, 1993). More recently XOR activity in terms of allopurinol-sensitive nitrite reductase activity has been demonstrated in human left internal mammary artery (Webb *et al.*, 2008b). Thus, these data together support the view that significant functional XOR likely exists in human arteries in addition to capillaries.

Interestingly, it seems that XOR is located within the cytoplasm (Jarasch *et al.*, 1981; Jarasch *et al.*, 1986) and on the outer cell membrane of human endothelial cells (Rouquette *et al.*, 1998). Whilst the source for the intracellular expression is clearly the endothelial cell itself, the cell membrane bound expression relates to XOR shed from organs such as the liver and, as evidenced from studies in rodents, in periods of metabolic stress also from the intestine (Yokoyama *et al.*, 1990; Terada *et al.*, 1992a). Circulating XOR binds with high affinity to endothelial cells via glycosaminoglycans (Adachi *et al.*, 1993; Radi *et al.*, 1997; Houston *et al.*, 1999). Once XOR has been released into the circulation, it is then free to be carried and bind to vascular endothelium, via these heparin sulphate proteoglycans, in distant parts of the circulatory tree. More recently, it has been demonstrated that not only is this binding occurring at the endothelial cell but also likely occurring at the level of the red blood cell too since using immunohistochemistry the presence of large amounts of XOR on the membrane of erythrocytes from healthy volunteers was demonstrated (Webb *et al.*, 2008b).

1.15.5 Upregulation of XOR in CVD

XOR activity/expression is upregulated in CVD. In animal models of CVD including atherosclerosis, hypercholesterolaemia and reperfusion injury, XOR expression/activity increases and blockade of activity improves disease burden/outcome (Grum *et al.*, 1986; Tan *et al.*, 1993; Nielsen *et al.*, 1994b; White *et al.*, 1996a). Similarly, in patients with CVD including coronary artery disease, hypertension and heart failure, both XOR expression and activity (in some cases identified as endothelium-bound activity) is

substantially increased (Spiekermann *et al.*, 2003b; Guzik *et al.*, 2006b; Landmesser *et al.*, 2007). In patients undergoing upper limb surgery (~90 min ischaemia), plasma XO activity in the ipsilateral arm was found to increase from ~2nmol/ml/min before reperfusion to ~10nmol/ml/min shortly after reperfusion, suggesting release of XO from ischaemic vascular endothelium (Friedl *et al.*, 1990). In addition to the stimulated release of XOR from organs such as the liver, expression/activity of XOR within endothelial cells can be upregulated by pro-inflammatory cytokines; specifically interferon gamma has been shown to elevate XDH levels in rat pulmonary artery and lung microvascular endothelial cells (Dupont *et al.*, 1992). Furthermore, evidence also suggests that hypoxia is a potent stimulus for XDH transcription. In rat pulmonary microvascular endothelial cells XDH mRNA levels were increased 2-fold in hypoxic [3% oxygen] relative to normoxic controls [20% oxygen] (Lanzillo *et al.*, 1996), although more moderate increases of ~50% have been demonstrated in bovine arterial endothelial cells using more moderate levels of hypoxia [10%] (Kelley *et al.*, 2006) or indeed no change in expression at all (in response to 0-3% oxygen conditions) has also been reported in these cells (Poss *et al.*, 1996). Interestingly in this latter study whilst no change in expression was observed, a doubling in activity was evident suggesting possibly post-translational alterations. Indeed, this possibility was confirmed in a study using cultured rat pulmonary microvascular endothelial cells in which hypoxia-induced post-translational phosphorylation of XDH was mediated by p38 kinase and casein kinase II (Kayyali *et al.*, 2001). Such observations might suggest that the multiple modes of upregulation of XOR permit a continuum for the enzyme whereby phosphorylation might provide an acute response to stimuli, such as hypoxia; over longer periods of time, the release and upregulation of protein synthesis maintains a role for this enzyme, in the face of prolonged ischaemic or inflammatory stimuli.

In all the above-mentioned CVD states whilst the changes in XOR expression and/or activity are deemed to be pathogenic, whether this pathogenic role relates to the activity of damaging superoxide or elevated uric acid generation is controversial and remains uncertain. In all of these disease states there is evidence that targeting XOR activity using either allopurinol or the more recently licensed non-purine based 2-(3-

cyano-4-isobutoxyphenyl)-4-methyl-5-thiazolecarboxylic acid (febuxostat), (Okamoto *et al.*, 2003; Tayar *et al.*, 2012) results in improvement of surrogate measures of disease severity, however there are a number of observations that have also limited enthusiasm for this approach to CVD due to an absence of beneficial effect or indeed a worsening. An underlying cause for these uncertain data may be due to the fact that inhibition of XOR will result not only in a decrease in both superoxide and uric acid generation but also inhibit the generation of beneficial NO from nitrite.

1.15.6 XOR in hypertension

A role for XOR in mediating the elevated blood pressure (BP) and associated 'endothelial dysfunction' in hypertension has been demonstrated in various animal models. Early experimental work assessing raised BP in the spontaneously hypertensive rat (SHR), the salt sensitive rat and in dexamethasone-induced hypertension in rats demonstrated that XOR expression and activity is upregulated in vivo in the kidney (Laakso *et al.*, 1998a; Laakso *et al.*, 2004). However, whilst allopurinol treatment completely blocked this elevated activity, it did not alter BP in these animals. In contrast, whilst others have also demonstrated elevated XOR activity in other tissues including the mesentery (Suzuki *et al.*, 1998b) and cremaster (Wallwork *et al.*, 2003) in the same models of hypertension i.e. the SHR and dexamethasone-induced hypertension respectively, the selective XOR inhibitor sodium 8-(3-methoxy-4-phenylsulfinylphenyl)pyrazolo[1,5-α]-1,3,5-triazine-4-olate monohydrate (BOF-4272) or allopurinol did reduce BP. These pre-clinical studies have been followed up by others and rather than clearing up these anomalies continued investigation has fuelled the controversy further with equally opposing observations (Ong *et al.*, 2007; Sánchez-Lozada *et al.*, 2008; Szasz *et al.*, 2013). Numerous ideas have been put forward for these findings but these differences might simply reflect an unconsidered effect of inhibition of beneficial nitrite reduction.

In terms of the mechanisms involved in the pro-hypertensive effects of XOR, an increase in activity and/or expression will lead to an increase in uric acid and superoxide. It is elevation of the latter that, until recently, was thought to underlie the

role that XOR plays in both promoting raised BP and the endothelial dysfunction occurring in hypertension (Kojda *et al.*, 1999). Superoxide produced by XOR reacts with NO to form peroxynitrite (Beckman *et al.*, 1996b) and this scavenging of NO has been implicated in its reduced bioavailability in hypertensive states, and been shown to be reflected in measures of endothelial reactivity. In transgenic rats harbouring the human renin and human angiotensinogen genes, BP is elevated in an angiotensin II-dependent fashion (Ganten *et al.*, 1992). Endothelium- and NO-dependent vasorelaxation of renal arteries, isolated from such animals is impaired, however treatment of these vessels with oxypurinol reversed this effect, as also did treatment with superoxide dismutase (SOD), suggesting that XOR-derived superoxide was responsible for this effect. However, more recently there has been growing support for the view that the elevations in uric acid that characterize hypertension might have a more important role to play in the hypertensive pathology than previously suspected.

Hyperuricemia is associated with hypertension although whether it is a risk factor or a consequence remains unclear (Mazzali *et al.*, 2010). It has been postulated that uric acid might increase BP by two mechanisms; firstly an acute, reversible phase which involves an increase in the renin angiotensin system (Mazzali *et al.*, 2001); secondly a chronic, irreversible phase in which the renal vasculature is permanently damaged due to elevations in local urate levels leading to hypertension (Kang *et al.*, 2002). Uric acid has also been shown to reduce NO levels in bovine aortic endothelial cells and in a rat model of hyperuricemia, induced through administration of the uricase inhibitor, oxonic acid (Khosla *et al.*, 2005). It is possible therefore that both uric acid and superoxide have a role to play in reducing the bioavailability of NO. Importantly scavenging of NO by either of these pathways will result in less NO that enters the usual metabolism pathways of this molecule i.e. oxidation (Fukuto *et al.*, 2000). Evidence suggests that the levels of both the oxidative metabolites of NO, nitrite and nitrate, are lower in hypertensive individuals than in healthy volunteers; an effect the authors suggested in this study is a reflection of scavenging of NO due to oxidative stress (Forte *et al.*, 1997). However, what has not been previously considered is that the reduced levels of nitrite not only reflect reduced availability of NO generated

through the conventional L-arginine/NOS pathway but also reflect reduced substrate for NO generation via the activity of XOR.

1.15.7 Vasodilator and hypotensive effects of nitrite

Systemic nitrite administration causes dose-dependent increases in blood flow and accordingly decreases in BP in both healthy animals and volunteers (Vleeming *et al.*, 1997b; Cosby *et al.*, 2003b; Dejam *et al.*, 2007b; Petersson *et al.*, 2009b). In addition repeated nitrite administration (once daily dosing for 14 days), albeit intravenously administered and with supra-pharmacological dosing (12,000μg/Kg/min), lowered BP in primates (Dejam *et al.*, 2007b). Similarly, studies in healthy volunteers assessing the pharmacokinetics of nitrite infusion largely support the vascular studies in healthy volunteers demonstrating that very large doses of nitrite (achieving circulating concentrations >20μM) do effect BP decreases (Dejam *et al.*, 2007b; Pluta *et al.*, 2011). A recent review comparing physiological plasma nitrite concentrations across studies, in healthy volunteers, suggests levels fall within the 200-500 nM range (Grau *et al.*, 2007b). However, published values range from non-detectable or as low as ~20nM (Pannala *et al.*, 2003b) to as high as 5μM (Moshage *et al.*, 1995b). Such variability may reflect true levels in plasma but are also likely to reflect differences in techniques used for measurement of nitrite; an issue debated in several reviews (Pelletier *et al.*, 2006a; Grau *et al.*, 2007b; Tsikas, 2007a). Irrespective of this, these acute infusion studies suggest a requirement for high circulating levels of nitrite to bring about significant BP-lowering effects. More recently, an alternative approach to raising circulating nitrite levels has been taken in a bid to better and more easily investigate the effects of this anion on BP in humans and relies upon what has been termed the 'enterosalivary circuit' of nitrate.

1.16 The enterosalivary circuit and its role in nitrate reduction to nitrite

The enterosalivary circuit is required to facilitate the conversion of nitrate to nitrite. The bioactivation of orally ingested inorganic nitrate results in elevation of endogenous nitrite levels, a reaction that is facilitated by oral bacteria. This conversion is a complex process. After ingestion of a nitrate load, nitrate is rapidly absorbed from the proximal part of the gastrointestinal tract into the bloodstream (Mensinga *et al.*,

2003). In the bloodstream, nitrate from the diet mixes with endogenously generated nitrate, produced from the NOS pathway.

After dietary nitrate consumption there is a rapid rise in plasma nitrate concentration with rises in nitrate evident within 30 minutes (Webb *et al.*, 2008c; Kapil *et al.*, 2010). This elevation peaks at 1.5 hours and remains elevated for up to 6 hours after ingestion (Webb *et al.*, 2008c; Kapil *et al.*, 2010). Estimates suggest a half life between 6-8 hours (Wagner *et al.*, 1983; van Velzen *et al.*, 2008). Most nitrate (60-75%) is ultimately excreted in the urine with peak excretion occurring around 6 hours after ingestion (Wennmalm *et al.*, 1993; Pannala *et al.*, 2003a). However the remaining nitrate, up to 25%, is actively taken up by the salivary glands and secreted with the saliva resulting in concentrations of salivary nitrate being 10-20 times higher than plasma nitrate concentrations (Spiegelhalder *et al.*, 1976). In the oral cavity, commensal facultative bacteria reduce nitrate to nitrite (Doel *et al.*, 2005). A study in 1997 examined in much detail the nitrate-reducing bacteria on rat tongues. Rats were chosen as the model system since their tongues can be divided into 2 morphologically distinct zones, the anterior part, which has a smooth surface, and the posterior part that has deep clefts filled with bacterial biofilms. Up to 65% of bacteria were located in the deep clefts on the posterior surface of the tongue where only significant nitrite production was detected (Li *et al.*, 1997). The conversion of nitrate to nitrite within the oral cavity is reflected by rises in the levels of salivary nitrite in parallel with nitrate (Webb *et al.*, 2008c). However studies measuring oral nitrate reduction in healthy volunteers have confirmed this. Healthy volunteers treated with antiseptic mouthwash had a 90% reduction in oral nitrite production and a 25% reduction in plasma nitrite concentration. Interestingly the reduction in circulating nitrite concentration was concomitant with increased systolic and diastolic blood pressure (Kapil *et al.*, 2013).

In 2004 Lundberg and Govoni demonstrated that an oral load of sodium nitrate caused increases in circulating nitrite (Lundberg *et al.*, 2004). Further work has also demonstrated that plasma nitrite is significantly elevated after dietary nitrate consumption. This elevation peaked at 3 hours following ingestion and remained at

this level up until 5 hours. This slow and sustained manner of circulatory nitrite elevation was proposed to be reflective of the ingestion and enterosalivary processing of dietary nitrate (Webb *et al.*, 2008c). In support of the view that elevations of circulating nitrite following nitrate ingestion is dependent on the enterosalivary circuit, treatment of rodents or humans with antibacterial mouthwash as well as interruption of the enterosalivary circuit in humans by spitting, attenuate the increases in plasma, urinary and salivary nitrite after ingestion of a dietary nitrate load (Govoni *et al.*, 2008; Webb *et al.*, 2008c; Petersson *et al.*, 2009a). In actual fact this process of nitrate reduction may not be completely dependent on bacteria but may be a process facilitated by mammalian nitrate reductases (Jansson *et al.*, 2008). Indeed a recent study compared functional nitrate reductase activity in conventional and germ-free mice. Nitrate ($NaNO_3$, 10mg/kg) or placebo (NaCl) was given intraperitoneally. In germ-free animals receiving nitrate, plasma nitrite concentration was approximately 3-fold higher than conventional animals receiving nitrate. This pattern was also reflected in the liver, kidney and gastro-intestinal tissues. Interestingly, pretreatment with allopurinol (100mg/kg/day via the drinking water) for 3 days significantly attenuated circulating nitrite concentration by approximately 50%. This implicates XOR as potentially a key enzyme in mammalian nitrate reduction (Huang *et al.*, 2010). Reduction of nitrate to nitrite by mammalian nitrate reductases was shown in this study with elevations of nitrite noted in numerous tissues, however whether this correlates to human nitrate reductase activity in nitrate processing in the oral cavity is not fully understood. The possibility of mammalian nitrate reductases in the oral cavity contributing to nitrate processing should not be completely disregarded.

Following nitrate reduction in the oral cavity salivary nitrite is swallowed and in the acidic environment of the stomach is protonated to form nitrous acid (HNO_2), which decomposes further to form NO and other nitrogen oxides (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994). However, some of that nitrite is thought to escape the acidic environs of the stomach and enter the blood stream. Nitrite reduction to NO is greatly enhanced by reducing compounds such as vitamin C and polyphenols, both found

abundantly in the diet (Peri *et al.*, 2005; Gago *et al.*, 2007). A schematic providing an overview of the enterosalivary circuit is shown in figure 1.7.

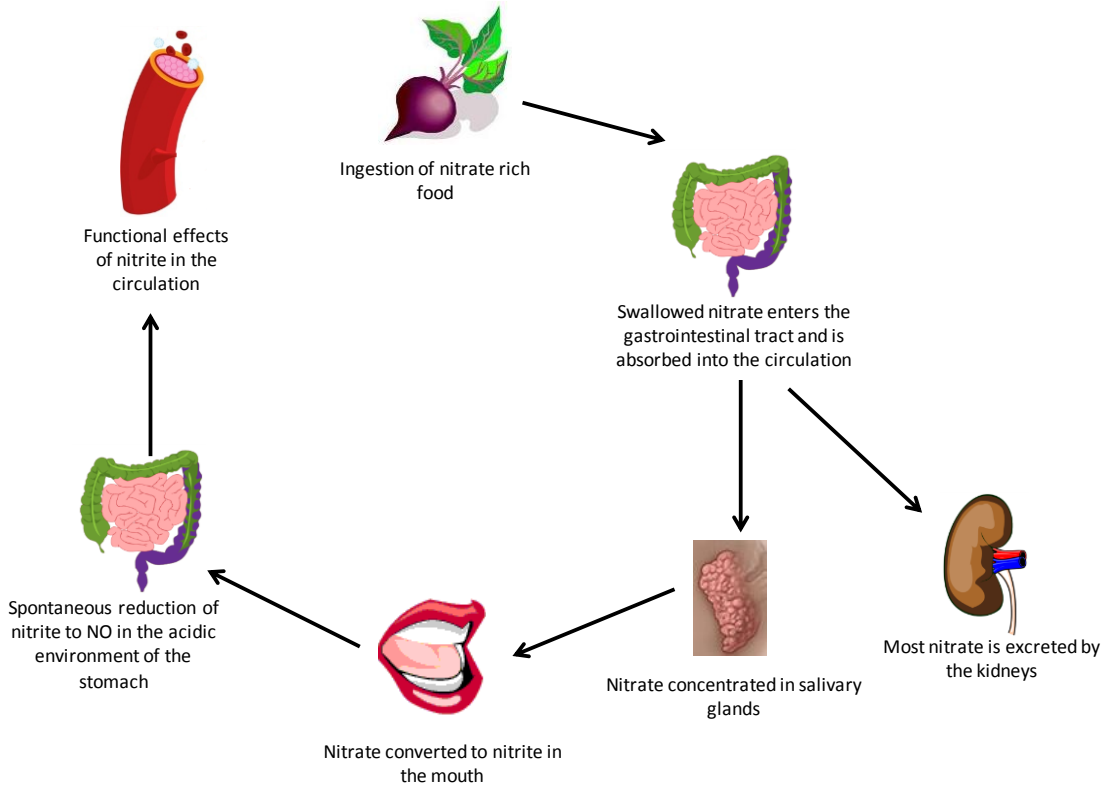


Figure 1.9: The enterosalivary circulation showing the conversions and locations that occur in the activation of nitrate to nitrite and nitrite to NO.

1.17 Sources of nitrate and nitrite

In the body there are 2 main sources of nitrate and nitrite, the diet and endogenous NO generation. The L-arginine/NOS pathway contributes through the rapid oxidation of NO to nitrate and nitrite. In the circulation NO oxidation is facilitated by the multicopper oxidase, ceruloplasmain (Shiva *et al.*, 2006). However, nitrate is the dominant final oxidation product in plasma with concentrations (micromolar) normally at least 2 orders of magnitude higher than nitrite (nanomolar) (Moncada *et al.*, 1991). In eNOS KO mice, plasma concentrations of nitrite are reduced by up to 70% (Kleinbongard *et al.*, 2003). The other major source of nitrate, and to a lesser extent nitrite, is our everyday diet. Vegetables are the predominant daily dietary source of nitrate (~80-85%) (Susin *et al.*, 2006; Hord *et al.*, 2009), and cured meats contain some nitrite, which is commonly used as a preservative against bacterial contamination as

well as a colour enhancer (Lundberg, 2009). Green leafy vegetables e.g. spinach, rocket and beetroot, are particularly high in nitrate and 1 serving of such a vegetable contains more nitrate than what is endogenously formed by all the 3 NOS isoforms during a day (Lundberg *et al.*, 2009).

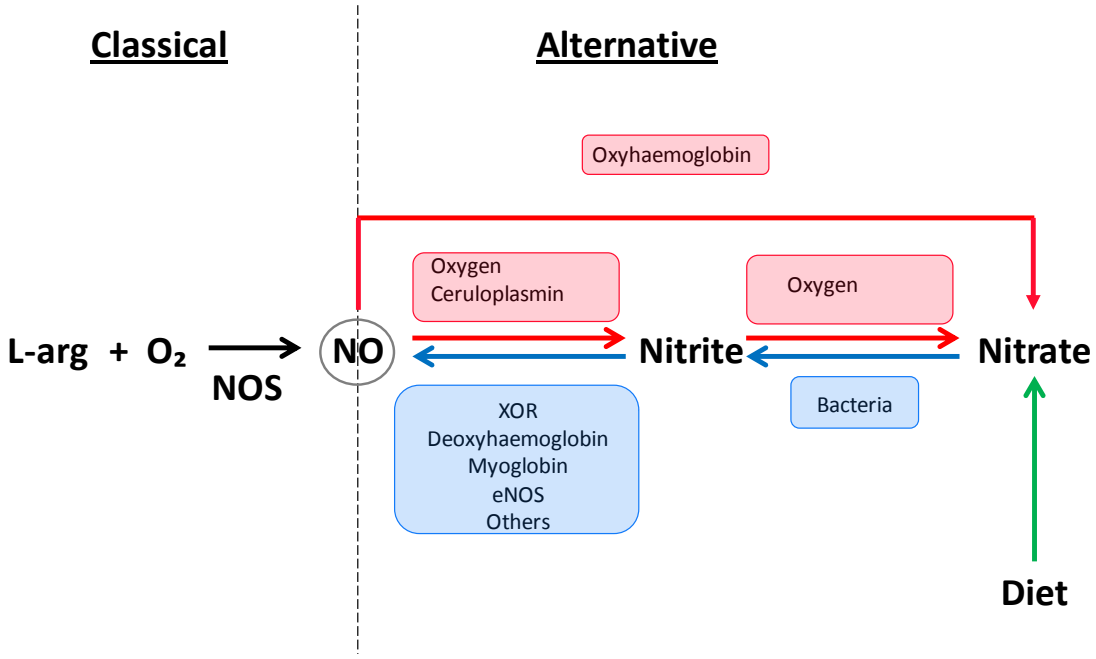


Figure 1.10: The L-arginine:NOS pathway and the nitrate-nitrite pathway of NO generation. The L-arginine:NOS pathway requires molecular oxygen to produce NO. The nitrate-nitrite pathway is gradually activated as oxygen tension falls therefore can be viewed as a back-up system to ensure that there is sufficient NO formation when oxygen supply is limited.

1.18 Effect of elevation of nitrite by a dietary nitrate strategy

In 2006 Larsen et al. first demonstrated the functional effects on the cardiovascular system of elevating plasma nitrite concentration using nitrate. They observed that in 17 physically active, healthy volunteers, a 3-day dietary nitrate supplementation significantly reduced diastolic blood pressure and mean arterial pressure by 3.7 and 3.2mmHg respectively (Larsen *et al.*, 2006). These effects were coupled to increases in plasma nitrate (placebo=26±11µM; post nitrate ingestion=178±51µM) and plasma nitrite (placebo=0.138±0.038µM; post nitrate ingestion=219±105µM). The daily nitrate dose used in the study was equivalent to the amount found in 150 to 250g of a nitrate-rich vegetable such as spinach, beetroot or lettuce (Larsen *et al.*, 2006). Since this work the beneficial effects of dietary nitrate supplementation, through conversion to nitrite, have been shown in numerous studies. These beneficial actions are not just limited to

lowering blood pressure but also include effects on platelet reactivity as well endothelial function.

Work from our group showed that ingestion of a dietary nitrate load, this time in the form of 500ml beetroot juice (45.0±2.6mM) significantly lowered blood pressure (Webb *et al.*, 2008c). This effect was correlated with rises in plasma nitrite concentration. In addition endothelial function was assessed by measuring brachial artery diameter (in the nondominant arm) in response to the endothelium-dependent reactive hyperaemia response before and after an ischaemic insult (Webb *et al.*, 2008c). Nitrate ingestion prevented ischaemia-induced endothelial dysfunction. Furthermore, in this study it was shown that nitrate intake attenuated the platelet aggregation response induced by ADP or collagen. These effects were mediated through nitrite since prevention of nitrite elevation in the circulation (by blocking the enterosalivary circuit by asking volunteers to spit out saliva before juice intake) abolished the beneficial effects seen (Webb *et al.*, 2008c).

Using nitrate salt supplementation (a potassium nitrate (KNO_3) capsule) these effects were shown to be dose-dependent with 4mmol being a threshold dose in terms of rise in plasma nitrite concentration (1.3 fold) and blood pressure decrease (Kapil *et al.*, 2010). In this study the rise in plasma nitrite was shown to be accompanied by a rise in cGMP the most sensitive indicator of NO bioactivity thereby providing strong evidence for the generation of bioactive NO (Kapil *et al.*, 2010). The peak effect on BP reduction was with the highest dose of inorganic nitrate tested i.e. 24mmol (9.4±1.6mmHg for SBP, 6.0±1.1mmHg for DBP). Post hoc analyses in this study demonstrated that the magnitude of the blood pressure response was directly related to the baseline blood pressure i.e. the higher the baseline blood pressure the greater the peak in blood pressure reduction achieved. This analyses uncovered the potential benefit that nitrate might be more effective when it is required i.e. in people with a high normal blood pressure or hypertension (Kapil *et al.*, 2010).

1.19 Aims

Recently, it has emerged that the NO metabolites, nitrite and nitrate can be chemically reduced in vivo to biologically active NO. This novel means of generating cytoprotective NO depends on reduction of nitrate to nitrite by facultative anaerobes on the dorsal surface of the tongue, entry of the nitrite into the enterosalivary circulation, transit to the stomach and absorption through the gut wall (Lundberg *et al.*, 2008). Conversion of nitrite to NO is then facilitated by a family of proteins that exhibit 'nitrite reductase' activity including the globins (Basu *et al.*, 2007; Hendgen-Cotta *et al.*, 2008; Tiso *et al.*, 2011), eNOS (Gautier *et al.*, 2006; Webb *et al.*, 2008a) and XOR (Zhang *et al.*, 1998a; Li *et al.*, 2001b; Webb *et al.*, 2004a). The nitrate-nitrite-NO pathway has been shown to exert a number of beneficial effects, including lowering systemic blood pressure and protecting against I/R injury (Webb *et al.*, 2004a; Larsen *et al.*, 2006; Hendgen-Cotta *et al.*, 2008; Webb *et al.*, 2008c). Indeed, ingestion of (inorganic) nitrate may underlie the cardioprotective phenotype of a diet rich in fruits and vegetables (Lundberg *et al.*, 2006; Kapil *et al.*, 2010).

In the context of disease it is unknown whether the distribution of nitrate and nitrite is affected by CVD and if so to what extent, therefore in my PhD I have investigated whether it is possible to elevate circulating (and tissue) concentrations of nitrite through dietary nitrate or dietary nitrite supplementation. In addition I have investigated whether or not nitrite reductase pathways are altered by CVD. Finally, through its reduction to nitrite and thence NO I have investigated whether dietary nitrate might be used as a strategy to alter the inflammatory response therefore my specific aims were:

1. To determine whether the distribution of nitrite and nitrate differs between healthy animals and animals with hypertension or atherosclerosis.
2. To determine whether the nitrite reductase pathways within the cardiovascular system differ in health and disease.
3. To determine whether dietary nitrate might influence disease outcome by modulating inflammatory responses.

Chapter 2:

Methods

2.1 Animals

2.1.1 Mice

All experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986, UK and approved by the UK Home Office. Male mice 12-14 weeks were used in all experiments. C57BL/6J wild-type (WT) and apolipoprotein E (ApoE) knockout (KO) (on a C57BL/6J background) mice breeding pairs were purchased from Charles River and mice bred in house.

Heterozygotic mice with targeted disruption of 1 ApoE allele were brother-sister mated to give offspring deficient in ApoE. Genotype was confirmed by polymerase chain reaction (PCR). The ApoE KO strain was chosen as the defect renders the mice incapable of producing ApoE, a glycoprotein essential for the transport and metabolism of lipids (Plump *et al.*, 1992). ApoE KO mice have been shown to develop lesions of atherosclerosis similar in appearance and distribution to those found in humans (Nakashima *et al.*, 1994). All animals were fed a standard chow diet (Special Diet Services, Rat and Mouse No.1 Maintenance), were allowed water ad libitum and kept on a normal 12/12 light cycle.

2.1.2 Rats

Spontaneously hypertensive rats (SHR) were first developed in 1963 by Okamoto and Aoki (Okamoto *et al.*, 1963). A male Wistar strain rat with persistent high blood pressure (150 to 175mmHg) showing spontaneous hypertension was mated with a female rat (blood pressure range 130 to 140mmHg) of the same strain. From the subsequent generations the SHR model was developed (Pinto *et al.*, 1998). SHR and Wistar Kyoto (WKY) controls (14-16 weeks) were purchased from Charles River, UK. The raised blood pressure of the SHR and WKY animals used in this study were confirmed by blood pressure measurements in anaesthetized animals made in our laboratory by Dr Bubb, giving mean arterial pressure of 126.8±3.3mmHg for SHR (n=16) and 95.6±3.6mmHg for WKY (n=12). Animals were kept for 1 week for acclimatisation prior to use.

2.2 Genotyping

2.2.1 Tissue processing

To confirm the genetic status of the ApoE KO and WT mice genotyping was conducted. Mouse tail snips (1cm length) were cut into several pieces and DNA was extracted using a commercially available kit (Qiagen, UK). Tissue lysis buffer (ATL; 180µl) and 20µl proteinase K (>600mAU/ml) was added to tail snips and left to incubate overnight (16h) in a water bath at 56°C. Samples were then mixed with 200µl lysis buffer (AL) and 200µl ethanol (96-100%). The resultant mixture was pipetted into DNeasy mini spin columns placed in a 2ml collection tube. The column was then centrifuged at 8,000g for 1min following which the collection tube and flow through were discarded. The mini spin column was then placed into a new collection tube and 500µl wash buffer 1 (AW1 diluted in 96-100% ethanol) was added. This was then centrifuged for 1min at 6,000g. The flow through and collection tube were again discarded and the mini spin column placed into a new collection tube, 500µl wash buffer 2 (AW2 diluted in 96-100% ethanol) was added and the tube centrifuged for 3min at 20,000g to dry the DNeasy membrane. The flow through and collection tube were discarded. A final elution step was conducted where the mini spin column was placed into a 1.5ml microcentrifuge tube, 200µl of buffer AE (Elution Buffer) added to the spin column membrane and incubated at room temperature for 1min followed by a final spin for 1min at 6,000g. The final elution step was repeated to increase overall DNA yield.

2.2.2 DNA quantification

DNA was quantified using the nanodrop ND-1000 UV/visible spectrophotometer (NanoDrop Technologies, USA), the device being controlled by computer-run ND-1000 software version 3.3.0. DNA absorbs ultra violet (UV) light and this property is used as the basis for quantification. To measure DNA concentration (and purity) the intensity of absorbance of DNA was measured at 260nm (A_{260}) and 280nm (A_{280}). DNA quantity was measured on the basis that a pure DNA sample has an A_{260}/A_{280} ratio of 1.8. Any samples with a ratio lower than this were excluded. For measurement, 2µl of each sample was placed into the measuring point and after calibration of the nanodrop an automated ratio and ng/µl concentration were provided.

2.2.3 Polymerase chain reaction

Polymerase chain reaction (PCR) amplifies a specific segment of DNA multiple times so that the DNA segment can be visualized on a gel to confirm the absence/presence of the gene of interest. There are 4 steps of the PCR:

1. *Denaturation step* - DNA is heated to high temperatures causing the hydrogen bonds between strands to break, therefore the DNA 'melts' and single stranded DNA (ssDNA) is yielded.
2. *Annealing step* - the reaction temperature is lowered to assist the annealing ('joining') of primers to their target sequences on the DNA template.
3. *Extension/elongation step* - formation of new complimentary strands of DNA, a reaction catalysed by DNA polymerase.
4. *Final elongation* - this step is required to ensure extension of any remaining ssDNA.

The ApoE gene contains 4 exons and is located on mouse chromosome 7. In ApoE KO mice the gene has been disrupted by insertion of a neomycin cassette sequence. The sequence replaces exon 2 and a part of exon 3 (Piedrahita *et al.*, 1992). Determination of the presence of ApoE or the neomycin cassette was utilised to distinguish between ApoE KO and WT mice. Specific forward and reverse primers were used (Table 2.1). A master mix of 10X PCR buffer (Applied Biosystems), deoxynucleoside triphosphates (dNTPS - Invitrogen), primers (Integrated DNA Technologies – IDT) and TaqPolymerase (Taq - Applied Biosystems) was made. The volume of each reaction component required for a single reaction is shown in table 2.2. For each reaction, 10µl of master mix was added to 2µl of DNA. The cycling conditions for the PCR are shown in table 2.3.

Primer	Sequence 5' --> 3'	Primer Type	PCR product size, base pairs (bp)
oIMR0180	GCC TAG CCG AGG GAG AGC CG	Common Forward	Heterozygote ~ 155 and ~ 245
oIMR0181	TGT GAC TTG GGA GCT CTG CAG C	Wild type Reverse	WT ~ 155
oIMR0182	GCC GCC CCG ACT GCA TCT	Mutant Reverse	ApoE KO ~ 245

Table 2.1: The gene specific primer sequences that were used in the PCR amplifications.

Reaction Components	Volume for 1 reaction/µl
10X PCR buffer	1
Magnesium chloride (MgCl2) 25mM	0.8
dNTPs 5mM	0.4
Forward (F) Primer 10µM	0.5
Reverse (R) Primer 10µM	0.5
Forward (F) Primer2 10µM	0.5
Taq 5U/µl	0.05
Water	5.25

Table 2.2: List of the reaction components in the PCR master mix required for 1 reaction.

Step	Temperature	Time	Note
1	94°C	10min	
2	94°C	30s	
3	68°C	40s	
4	72°C	1min	Repeat steps 2-4 for 35 cycles
5	72°C	2min	
6	10°C		hold

Table 2.3: The cycling times and conditions used for the amplification of the ApoE region of DNA as used on the Peltier Thermal Cycler (MJ Research).

2.2.4 Visualization of PCR products

PCR products were resolved by agarose gel electrophoresis. A 1.5% wt/vol agarose gel (multi-purpose molecular grade; Bioline, UK) was dissolved by heating in 1X tris-borate-EDTA (TBE) buffer (0.01M Tris base; 0.01M Boric acid; 0.004M EDTA – pH 8.0) mixed with GelRed (Biotium - 10µg/ml) and then allowed to set. In a cast 10µl of sample was added to 2µl of 6X DNA coloured loading buffer (GibcoBRL - 3g Ficoll 400; 4ml EDTA, pH 8.0; 5ml 1% bromophenol blue; made up to 20ml dH_2O). A ladder (4µl of a 100bp) was run and the PCR bands were visualised using a High Performance Ultraviolet Transilluminator (Bioimaging Systems). Figure 2.1 shows a representative image of the analysis of PCR products from DNA of ApoE KO and WT mice.

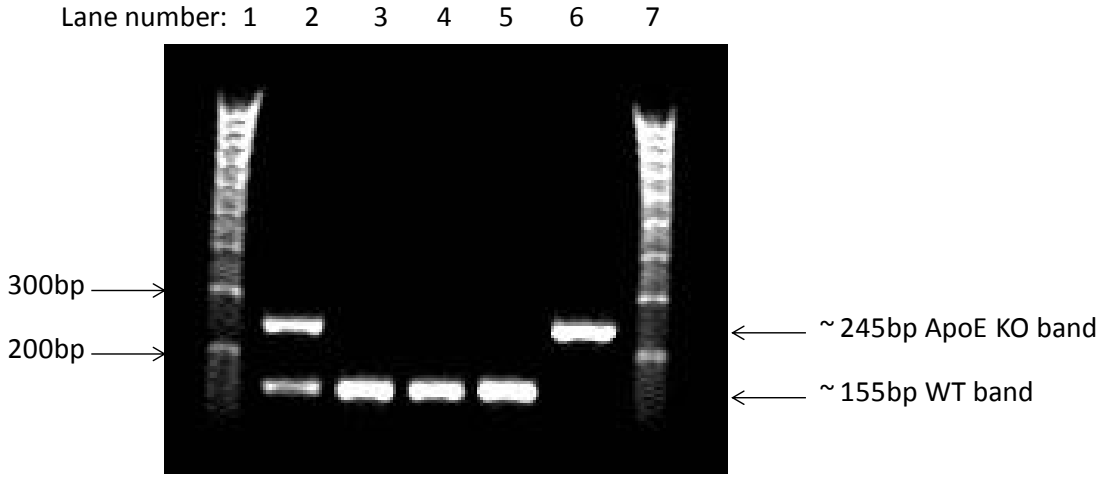


Figure 2.1: An example gel from electrophoretic analysis of PCR products from genomic DNA of WT mice and ApoE KO mice. Lanes 1 and 7 represent a 100 bp ladder with the 300 and 200 bp markers shown, lane 2 a heterozygote, lanes 3, 4 and 5 a WT mouse whilst lane 6 represents the band for an ApoE KO mouse.

2.2.5 Dietary nitrate supplementation

WT mice were randomly assigned to 1 of 6 different treatment groups and ApoE KO mice were split into 1 of 3 groups (table 2.4):

WT mice	ApoE KO mice
Control	Control
KNO_2 0.6mM	KNO_2 0.6mM
KNO_2 1.0mM	KNO_3 15mM
KNO_3 0.5mM	
KNO_3 15mM	
KNO_3 45mM	

Table 2.4: The list of treatment groups WT and ApoE KO mice were assigned to.

For dietary nitrate supplementation potassium nitrite (KNO_2, Sigma-Aldrich, UK) or potassium nitrate (KNO_3, Sigma-Aldrich, UK) was dissolved in 1 litre (L) of 18MΩ dH_2O. This dH_2O had undetectable (<50nmol/L) levels of nitrite or nitrate. The drinking water was made fresh and replaced every 2 days for 2 weeks.

2.3 Sample collection

2.3.1 Blood and tissue collection

Blood and tissue were collected from animals for nitrite and nitrate (collectively termed NO_x) analyses. Animals were anaesthetized using isoflurane (2%, vapourised in 100% oxygen at 0.4L/min, Baxter, UK). Blood was extracted by cardiac puncture into heparin (25units/ml of blood) or citrate (0.109M sodium citrate) coated 23-gauge needles and immediately centrifuged at 4°C, 12,000 g for 5min. The plasma and blood were separated and both were snap frozen in liquid nitrogen until use.

Following collection of blood the left ventricle of the heart was exposed and a cannula inserted and the animal then perfused with phosphate buffered saline (PBS, Sigma-Aldrich, UK) at a pressure of 100mmHg, until all blood had been flushed from the system. The internal organs (heart, lungs, liver, kidneys, spleen, mesentery and aorta) were then harvested and snap frozen in liquid nitrogen. All samples were stored at -80°C until use for biochemical analyses.

2.3.2 RBC collection

Blood was immediately centrifuged at 1,600g for 5min at room temperature. The plasma and buffy coat layer were removed, the plasma aliquoted and snap frozen in liquid nitrogen. RBCs were washed in PBS and the centrifuge/wash process repeated to generate a compacted RBC pellet.

2.4 Preparation of samples for biochemical analyses

2.4.1 Plasma preparation for NO_x analysis

In order to measure only circulating free NO_x levels all plasma samples were deproteinated. This is necessary for several reasons. Firstly during measurement of NO_x the bubbling of protein-containing samples results in frothing. Secondly, it is possible that nitrite will react to form either RSNO or RNNO groups and deproteination prevents this.

For deproteination plasma samples were centrifuged at 4°C, 14,000g for 60min through Microcon® Ultracel YM-3 (3kDa) filters (Millipore Corporation, Billerica, USA). The filters were then discarded and the filtrate snap frozen in liquid nitrogen and the samples stored at -80°C until use for analysis. Prior to use all filters were washed 2 times with low NOx containing 18MΩ dH_2O to remove any potential contaminants prior to addition of the plasma sample.

2.4.2 Tissue preparation for NO_x analysis

Prior to homogenisation each organ was weighed and then diluted with a protease inhibitor cocktail made in PBS (table 2.5). Using a Precellys homogenizer (Bertin Technologies, France) samples were then homogenised at 4°C. The homogenate was then centrifuged at 4°C, 10,000g for 5min. Protein concentration of the resultant supernatant was determined (see section 2.4.3) before being passed through a washed microcon YM-3 filter at 4°C, 14,000g for 90min. The filters were discarded and the filtrates snap frozen and stored at -80°C until use.

2.4.3 Protein determination

Protein concentration of tissue supernatant was determined using a Bradford protein assay (Bradford, 1976). A standard curve was generated from bovine serum albumin (BSA, 2mg/ml) by serial dilution at 0.0625-1mg/ml (figure 2.2) and then 10µl of each standard and 10µl of each sample was loaded in duplicate on a 96-well plate. Reagent (200µl; Bio-Rad, UK; diluted 1:5 in MQ H_2O) was then added to each of the wells. The light absorbance was measured using a spectrophotometric plate reader (MRX-TC Revelation, Dynex Technologies, UK) at wavelength 570nm. The protein concentration was determined by comparing the light absorbance of the unknown samples to the standards.

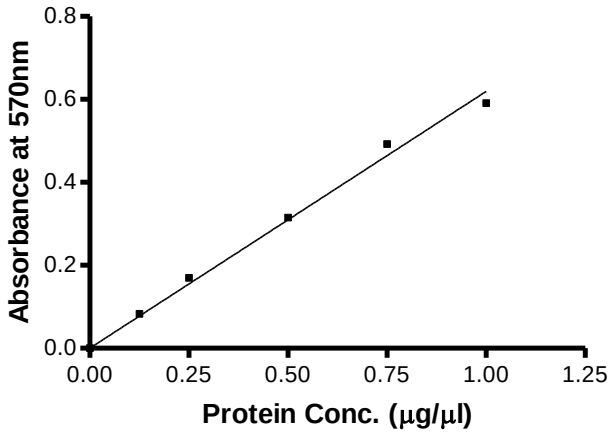


Figure 2.2: Typical standard curve generated from the Bradford protein assay.

Inhibitor	Target	Stock conc.	Made up in	Final conc.
Benzamidine	Potent inhibitor of thrombin and trypsin	1mg/ml (5.7mM)	water	1mg/L (5.7µM)
Antipain	Reversible inhibitor of serine and cysteine proteases. Inhibits papain and trypsin more specificity than leupeptin. Plasmin is inhibited only slightly. Also involved in inhibition of RNA synthesis	1mg/ml (1.5mM)	water	1mg/L (1.5µM)
Aprotinin	Basic single-chain polypeptide that inhibits numerous serine proteases by binding to the active site of the enzyme, forming tight complexes. It inhibits above all plasmin, kallikrein, trypsin, chymotrypsin and urokinase, but not carboxypeptidase A and B, papain, pepsin, subtilisin, thrombin and factor X. Used in cell culture to prevent proteolytic damage to cells and to extend lifetime of cells.	1mg/ml (0.15mM)	water	1mg/L
Leupeptin	Tripeptide aldehyde. Reversible competitive inhibitor of serine and cysteine proteases. Inhibits also phospholipase D and C activation in rat hepatocytes.	1mg/ml (2.1mM)	water	2mg/L (4.2µM)
Pepsatin A	Pentapeptide derivative. Reversible inhibitor of aspartic proteases, e.g. pepsin, cathepsin D, chymosin, renin	1.5mM (0.7mg/ml)	solubilised in 10% (v/v) acetic acid in methanol (9:1 methanol:acetic acid at 100%)	1µM
AEBSF	Irreversible inhibitor of Thrombin and other serine proteases. Inhibits by acylation of the active site of the enzyme. Much less toxic than PMSF and DFP	100mg/ml	water	400µM

Table 2.5: List of all protease inhibitors used to make up cocktail.

2.4.4 RBC preparation for NO_x measurement

RBCs were prepared according to published validated protocols (Dejam *et al.*, 2005). Briefly a compacted RBC pellet was resuspended 1:4 in nitrite preserving stop solution.

The stop solution consisted of 0.8M potassium ferricyanide ($K_3Fe(CN)_6$, Sigma, UK) and 0.1M N-ethylmaleimide (NEM, Sigma, UK) dissolved in 4.5ml MQ H_2O to which 500µl Nonidet P-40 (NP-40, Sigma, UK) was added. Ice-cold methanol was then added to the resultant mix in a 1:1 ratio to deproteinate the sample. The sample was then centrifuged at 13,000g for 5min at 4°C. The supernatant was collected and used for biochemical analyses.

2.5 Measurement of plasma, tissue and RBC nitrite and nitrate levels

To determine the concentration of nitrite and/or nitrate in a given sample the technique of ozone chemiluminescence was used. For this technique an NO analyser (NOA 280A, Sievers) was used to measure NO based on the gas-phase chemiluminescent reaction between NO and ozone (O_3). Essentially the levels of nitrite and nitrate in any sample are reduced to NO. Then NO is measured by the NOA and since for every one mole of nitrite or nitrate one mole of NO is generated one can assess accurately the levels of both anions in a given sample. When NO gas is mixed with ozone (O_3) it generates nitrogen dioxide in an excited state (equation 2.1).

$$NO + O_3 \longrightarrow NO_2^* + O_2$$

Equation 2.1: Reaction of NO with ozone (O_3) to produce excited nitrogen dioxide (NO_2^*).

The excess energy of NO_2^* can be taken up by reaction with other gas molecules (M, equation 2.2) or released as a photon (equation 2.3) when the NO_2^* spontaneously returns to a stable 'ground' state (NO_2), which releases energy as a photon ($h\nu$).

$$NO_2^* + M \longrightarrow NO_2 + M$$

Equation 2.2: Reaction of excited nitrogen dioxide (NO_2^*) with other molecules.

$$NO_2^* \longrightarrow NO_2 + h\nu$$

Equation 2.3: Stabilisation of excited nitrogen dioxide.

The release of the photon (emitted in proportion to the NO concentration) is detected by the NOA.

For sample measurements 2 main components are required, the purge vessel and the NOA (figure 2.3). An inert gas i.e. 100% N_2 bubbles through the reducing agent inside the purge vessel to purge any NO from the solution contained within the vessel. Standards and biological samples are added through the liquid sample inlet into the purge vessel where, depending upon the reducing agent, nitrite and/or nitrate is reduced to NO. The NO produced, which is in the gas phase, is then carried through the purge vessel to the NOA for quantification.

To determine the nitrite concentration samples were added to 0.09M potassium iodide (KI) in glacial acetic acid refluxing under N_2 at room temperature (equation 2.4). Initially a calibration curve is made based on the final dilutions from a stock solution of sodium nitrite ($NaNO_2$). Figure 2.4 shows an example trace that is obtained from different injection volumes and concentrations of standards. Figure 2.5 shows a calibration curve after integration of each peak to the correct standard.

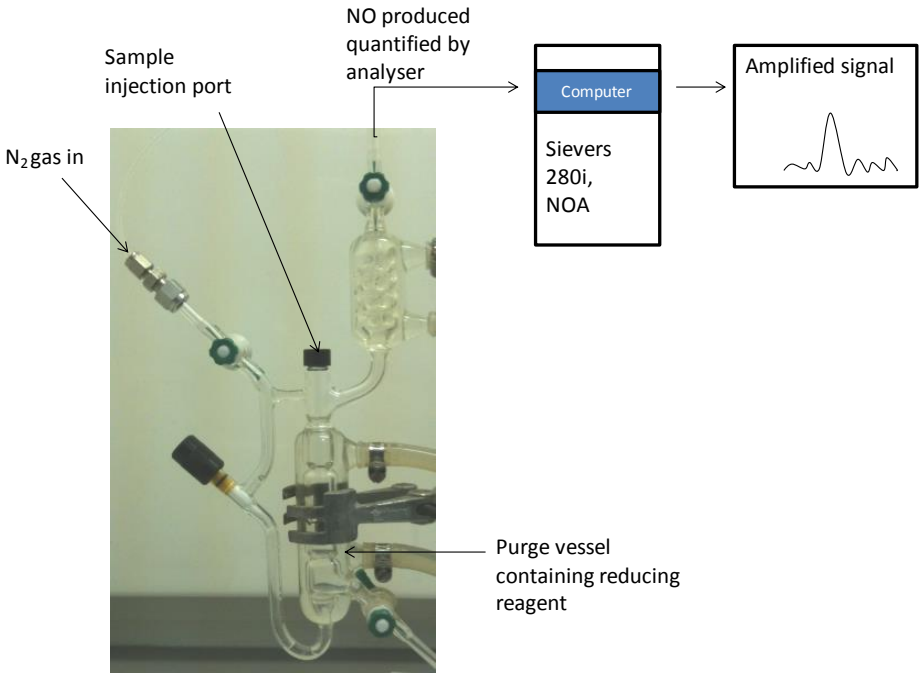


Figure 2.3: Diagram of the ozone chemiluminescence setup for nitrite determination.

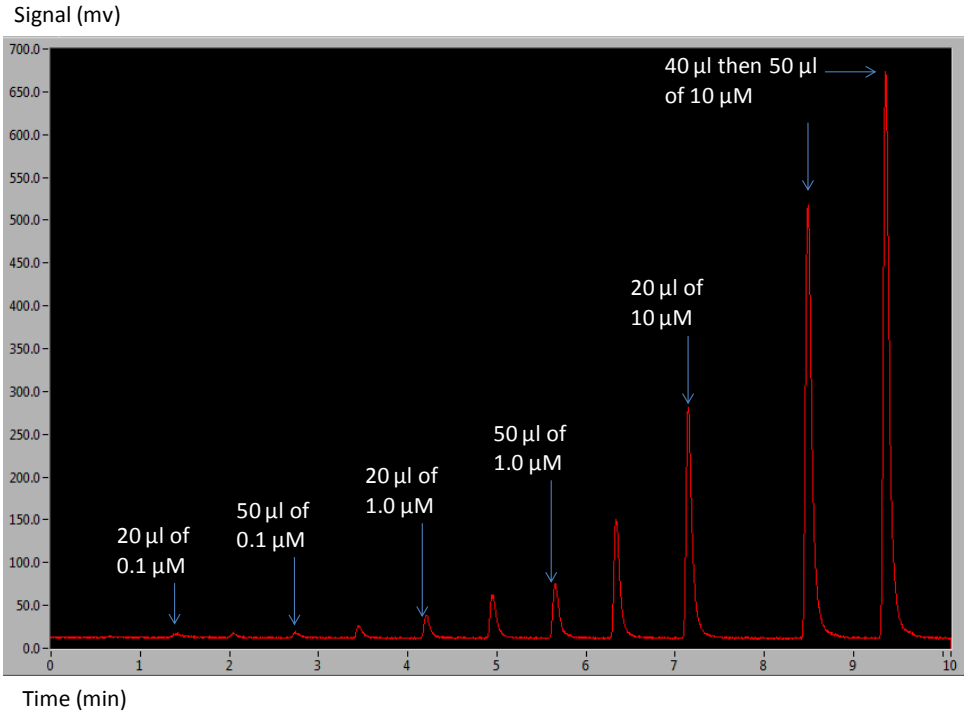


Figure 2.4: An example trace that is produced when standards of known concentration are injected into the purge vessel.

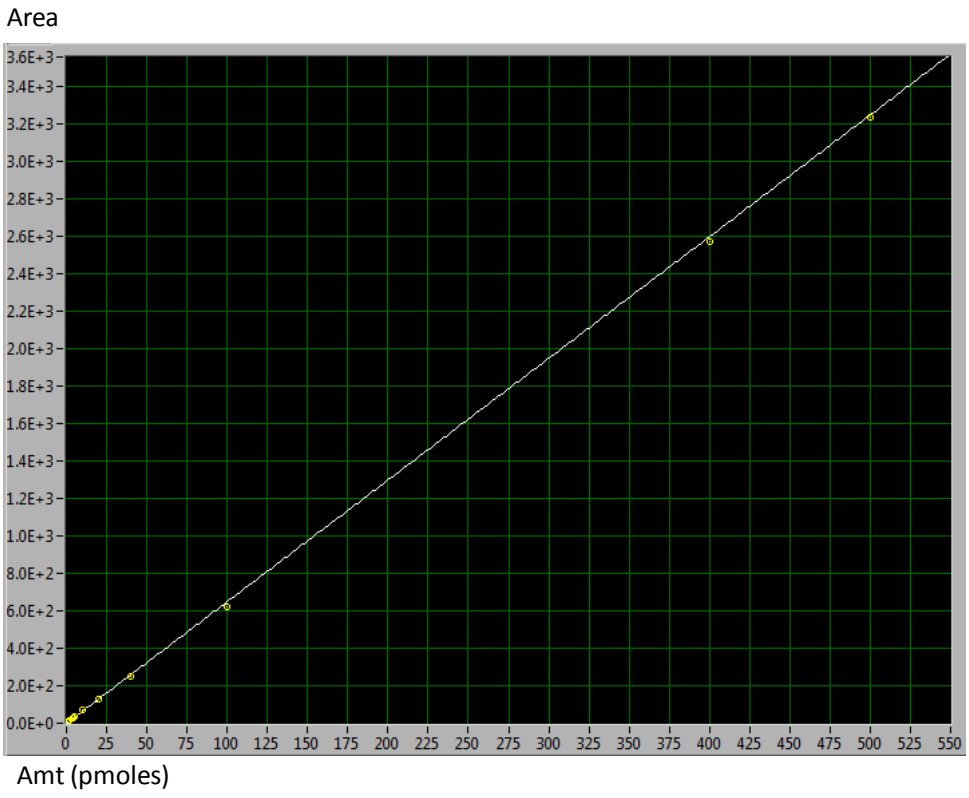


Figure 2.5: A typical standard curve produced prior to the measurement of nitrite in liquid samples (slope=6.5; intercept=-1.2; R^2=0.9999).

In the presence of protons (H^+) from the acid, nitrite (NO_2^-) is converted to the nitrosonium ion (NO^+, equation 2.4).

$$NO_2^- + 2H^+ \longrightarrow NO^+ + H_2O$$

Equation 2.4: Reaction of nitrite (NO_2^-) with protons (H^+).

The NO^+ associates rapidly with anions but in the presence of iodide (I^-) NO^+ is converted to NO via a nitrosyl iodide (ONI) intermediate (equation 2.5).

$$NO^+ + I^- \longrightarrow ONI$$

$$2ONI \longrightarrow 2NO + I_2$$

Equation 2.5: The conversion of the nitrosonium ion (NO^+) to NO via a nitrosyl iodide intermediate.

Thus the overall reaction chain for the reduction of 1 mole of nitrite gives one mole of NO (equation 2.6).

$$NO_2^- + 2H^+ + e^- \longrightarrow NO + H_2O$$

Equation 2.6: The overall reduction reaction of nitrite to NO.

These conditions are specific for the reduction of nitrite since the reduction of nitrate requires a much stronger reducing environment. The level of nitrate can be determined by placing samples in a much stronger reducing environment that will reduce all nitrate and nitrite. To measure the total NO_x samples were added to 0.1M vanadium (III) chloride (VCl_3) in 1M hydrochloric acid (HCl) refluxing under N_2 at 95°C to achieve high conversion efficiency. These conditions result in the reduction of all nitrate (equation 2.7) and nitrite (equation 2.6) to NO to give a total NOx value.

$$NO_3^- + 4H^+ + 3e^- \longrightarrow NO + 2H_2O$$

Equation 2.7: The overall reduction reaction of nitrate (NO_3^-) to NO.

By subtracting the amount of nitrite measured from the NO_x value a measure of the nitrate can be obtained. To accommodate the more powerful reducing environment required the setup is adjusted slightly (figure 2.6).

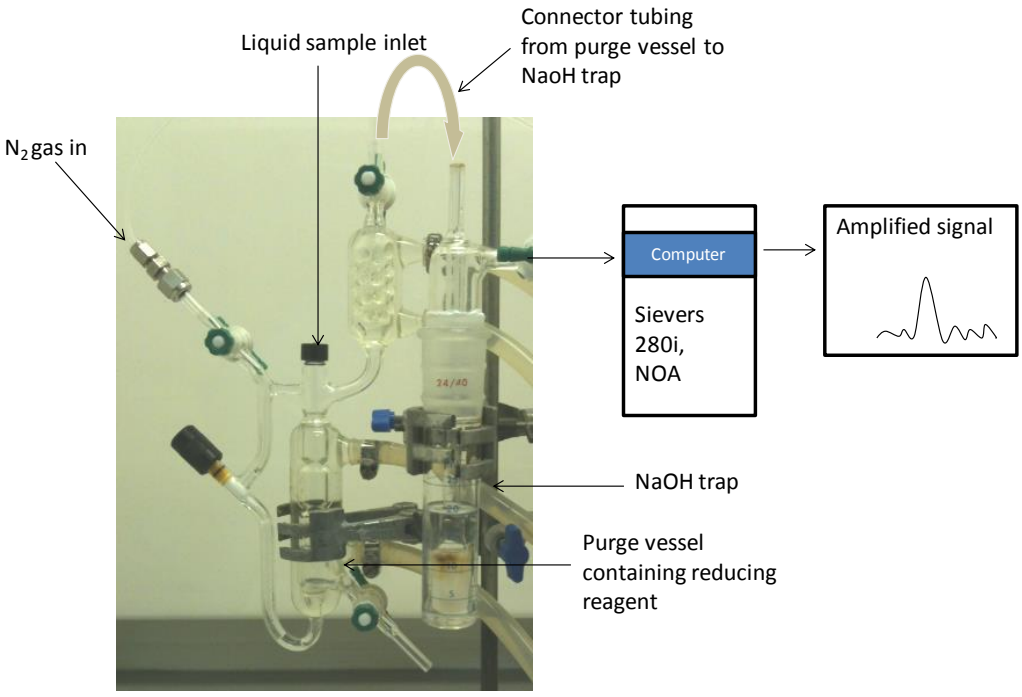


Figure 2.6: Diagram of the ozone chemiluminescence setup for NOx determination. A gas bubbler containing 1M sodium hydroxide (NaOH) was installed between the purge vessel and the NOA to prevent HCl vapour damaging the NOA.

2.5.1 Data analysis

For determination of NO_x levels in samples all plasma samples are expressed as a concentration whilst all tissue/RBC samples are normalized to protein concentration.

2.6 Measurement of nitrite reductase activity

Prior to use for nitrite reductase assessment protein concentration was determined using a Bradford protein assay (Bradford, 1976) on tissue supernatant or RBCs diluted 1:1000 (see section 2.4.3). For the measurement of the nitrite reductase activity of tissue and RBC samples gas phase chemiluminescence was used. Experiments were performed in a sealed 10ml glass reaction chamber containing citric acid-sodium phosphate dibasic (Na_2HPO_4) (Sigma, UK) buffer at pH 7.4 (physiological levels) or pH 6.8 (representing acidosis) and KNO_2 (10-300 µmol/l) in a total volume of 1ml (figure

2.7). Confirmation of correct pH was checked using a pH meter (Jenway, Staffordshire, UK). The pH meter was calibrated to solutions of known pH. The citric acid-Na_2HPO_4 solution was bubbled with 100% N_2 gas by means of an NO· scrubbing air filter (Sievers, Boulder, USA). The headspace NO· concentration was measured in parts per billion (ppb) by continuous sampling by ozone chemiluminescence (NOA 280A, Sievers).

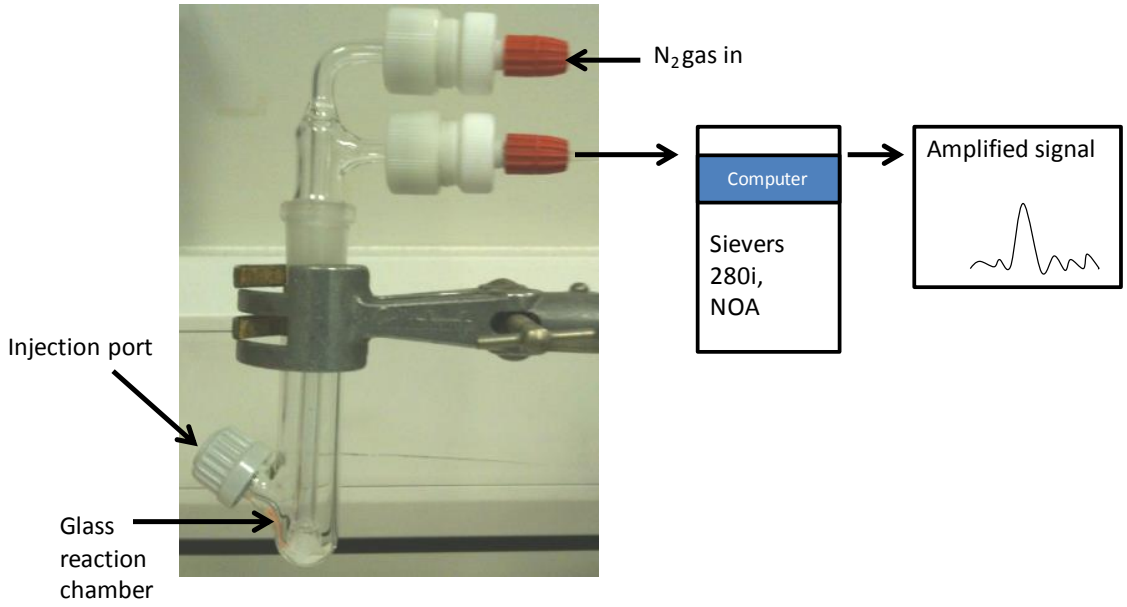


Figure 2.7: Diagram of the ozone chemiluminescence setup for the measurement of nitrite reductase activity.

In all experiments under each condition a baseline NO level was obtained prior to addition of 300-500µg of protein for tissues (calculated from the injection volume and protein concentration) or 20µl of RBC sample. The impact of biological tissue samples on NO production was determined firstly by adding washed RBCs/tissue supernatant to the glass reaction chamber, then adding nitrite and measuring NO production from nitrite over the following 2min. From this, the rate of NO· production (nmol per g of tissue per s) was calculated from the area under the curve (see figure 2.8 for a typical trace with just the addition of nitrite, figure 2.9 for a typical trace with just the addition of tissue supernatant and figure 2.10 with the addition of nitrite followed by the addition of tissue supernatant).

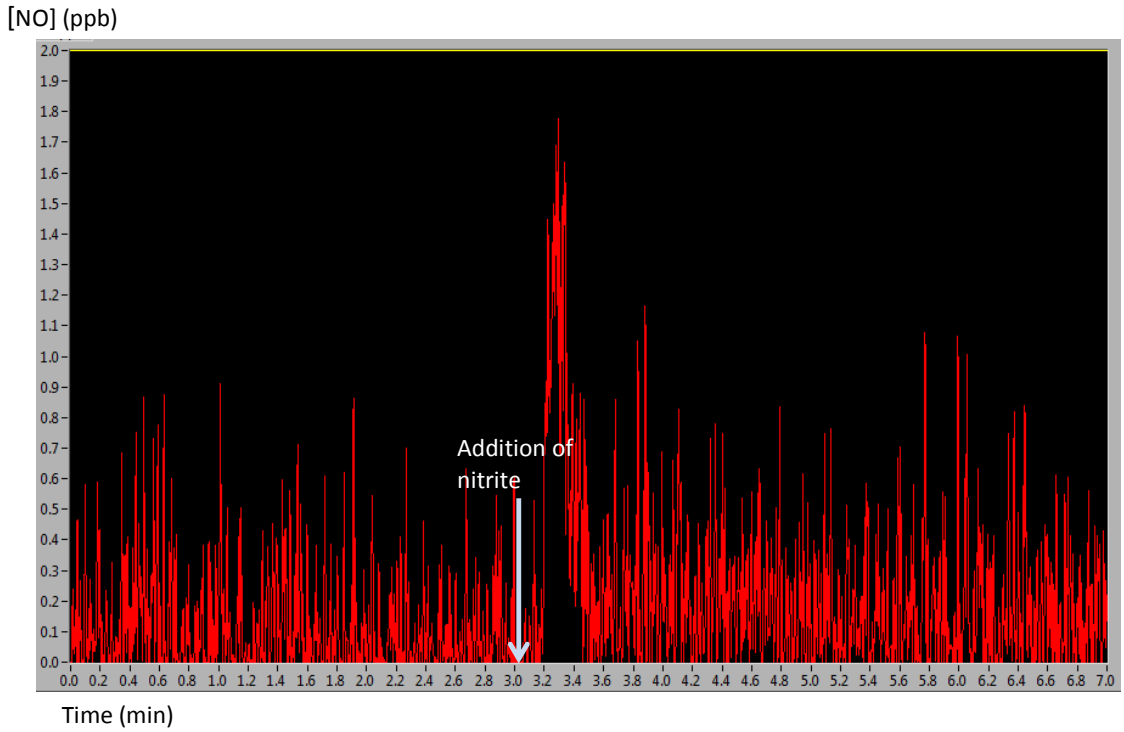


Figure 2.8: Typical trace of sampling of NO production following the addition of just nitrite (300µM) at pH 6.8 under anaerobic conditions.

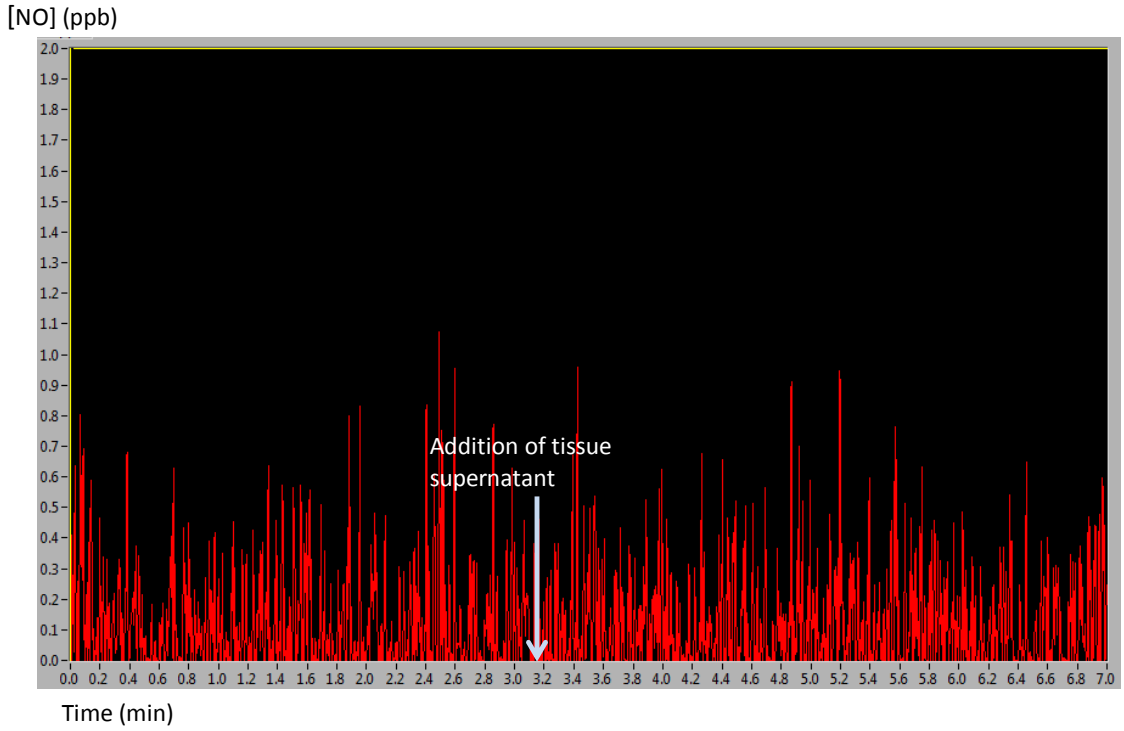


Figure 2.9: Typical trace of sampling of NO production following the addition of just tissue supernatant at pH 6.8 under anaerobic conditions.

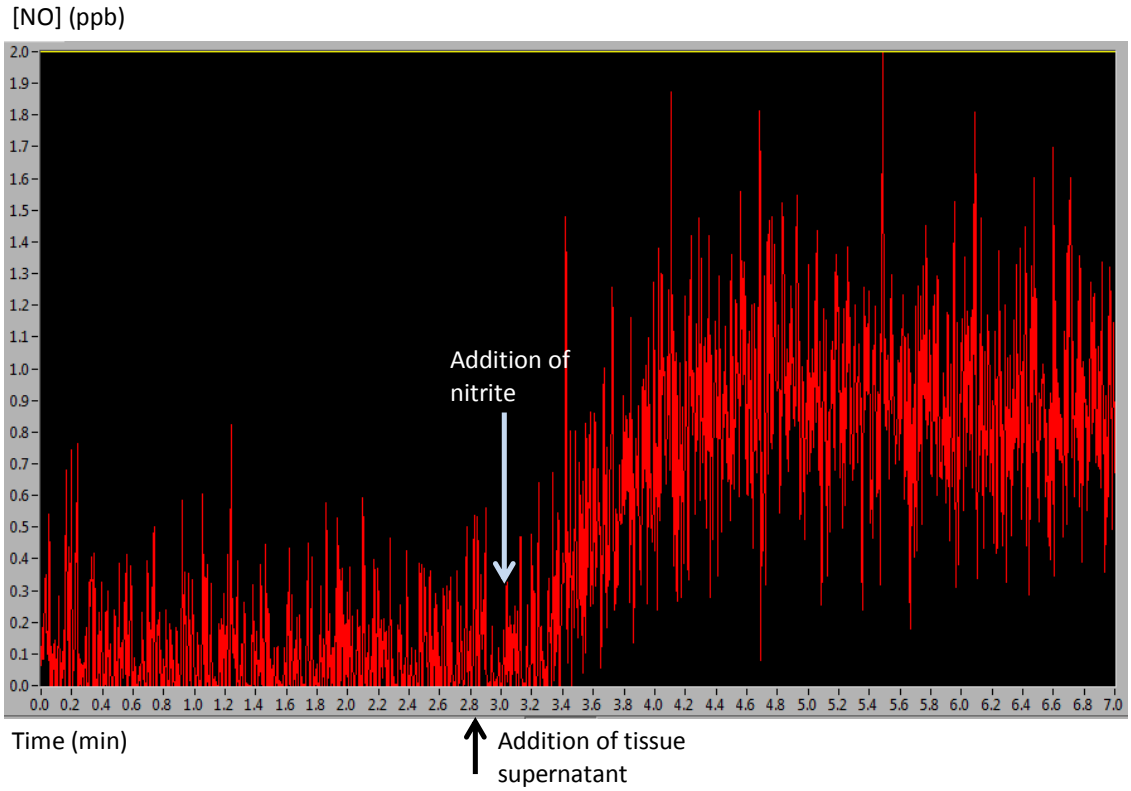


Figure 2.10: Typical trace of sampling of NO production following the addition of rat aorta supernatant and then nitrite (300µM) at pH 6.8 under anaerobic conditions.

To determine whether XOR might be involved in any nitrite reductase activity allopurinol (100µM) or vehicle (1M NaOH diluted in PBS) was incubated with the tissue sample for 30min prior to addition to the NO sampling chamber.

2.6.1 Data analysis

To normalize data to enable comparison between treatments, all data were corrected for protein amount therefore the rate of NO· production was converted from ppb per s to nmol per g per s:

The protein amount was calculated from the injection volume (µL) and the protein concentration (µg/µL) obtained from the protein assay. The protein amount was therefore the result of the values multiplied together to give a value in µg. As an example for a 300µg sample one would determine the reductase activity as follows -

The NOA samples at a rate of 0.228Lmin^{-1} and therefore 0.0038Ls^{-1}.

Under standard laboratory conditions (25°C and 1 atmosphere) 1 mole of an ideal gas has a volume of 24.47L, therefore the amount of pure gas in 1s=$\underline{0.0038}$=$1.5529x10^{-4}$ mol.

For 1ppb over 1s, 1ppb=$1x10^{-9}$ the rate of NO· production from 1ppb=$1.5529x10^{-4}x1x10^{-9}$.

Calculate rate=$1.5529x10^{-13}$mols^{-1}.

The value is then normalized for the amount of protein that was injected into the reaction chamber –

So for a 300µg sample the rate of NO· production=$1.5529x10^{-13} \div 3.0 \times 10^{-4}$

$= 0.5176x10^{-9}$molg^{-1}s^{-1}

2.7 Western blotting

Expression of XOR in washed RBCs was determined using western blotting. Western blotting of the abundant cytoskeletal housekeeping protein actin was also performed to estimate protein loading. Western blotting comprised of 3 main steps; sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), gel transfer and incubation and detection of proteins with the appropriate antibodies.

2.7.1 Sample Preparation

Cell pellets were lysed using a stock solution of tissue lysis buffer (table 2.6) and 0.5M NaF, 1% Triton X-100, 0.2M Na_3VO_4 as well as 1µg/ml each of the protease inhibitors benzamidine, aprotinin, antipain, leupeptin, pepstatin A and AEBSF.

Chemical	Final concentration (mM)	Action
10mM Tris-HCl	10	Buffer
50mM NaCl	50	Buffer
30mM $NaPP_i$	30	Excess phosphate, phosphatase inhibitor
2mM EDTA	2	Chelator of metal ions

Table 2.6: Composition of tissue lysis buffer.

2.7.2 Protein Determination

To quantify the amount of protein in each sample a Bradford Assay (Bradford, 1976) was performed as previously described (see section 2.4.3 Protein Determination). The protein concentration of each sample was then adjusted to 10µg/µl in tissue lysis buffer to enable equal amounts of protein to be loaded in each well of the gel.

2.7.3 SDS-PAGE

Samples were diluted 1 in 5 in Laemmli's loading buffer (final concentration 0.05M Tris-HCl, 6% glycerol, 0.002% bromophenol blue, 1.7% SDS and 1.55% DTT) and denatured by boiling at 95°C for 5min to break disulphide bonds therefore destroying the tertiary (and quaternary) structure, linearising protein in its primary structure so that proteins were separated by weight and charge.

2.7.4 Preparation of Gels

Precast gels, 10% Mini-PROTEAN® TGX™ (Bio-Rad, Hertfordshire, UK) were used. The precast gel was securely locked in to a casting stand. From the gel, the comb was removed and the casting stand was placed inside a Mini-PROTEAN® TGX™ Tetra System gel tank. Running buffer (25mM Tris-Base, 192mM glycine, 0.1% SDS) was then added to the gel tank until all wells were fully submerged and no air bubbles were present inside any well. Following addition of loading buffer 10µl of protein marker (Precision Plus Protein™ Dual Colour Standards, 10 - 250kDA, Bio-Rad, Hertfordshire, UK) was loaded in to the first well followed by 50µl of sample in to the subsequent wells and separated at 200V for 30min i.e. until the dye was visible at the bottom of the gel.

2.7.5 Transfer of Proteins

A piece of blot paper (PROTEAN®XL Size extra thick, Bio-Rad, Hertforshire, UK) and a Hybond™ Enhanced Chemiluminescence (ECL)™ nitrocellulose membrane (Amersham Biosciences, UK) were soaked in positive buffer (100ml methanol + 18.3g Tris-base, made up to 500ml with water) for 20min. Another piece of blot paper was soaked in negative buffer (100ml methanol + 1.5g Tris-base + 2.6g 6-amino-n-caprioic acid, made up to 500ml with water) for 20min. On to a platinum anode, the positive blot paper

was added followed by the nitrocellulose membrane, then the gel and finally the negative blot paper (figure 2.11). In order to ensure homogenous transfer of proteins the 'stack' was rolled to ensure removal of air bubbles. A cathode was then placed on to the 'stack' and the proteins were then transferred to the membrane by electrotransfer at 100mA using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad, Herforshire, UK) for 60min. Following this the membrane was stained with 0.1% Ponceau S Solution. Ponceau S reversibly stains proteins so that the membrane can be visually inspected to ensure equal loading and transfer. With distilled water the Ponceau S was washed 3-5 times with gentle agitation on a Luckham R100 Rotatest Shaker (Ecomat, Reading, UK) for 3min per wash step.

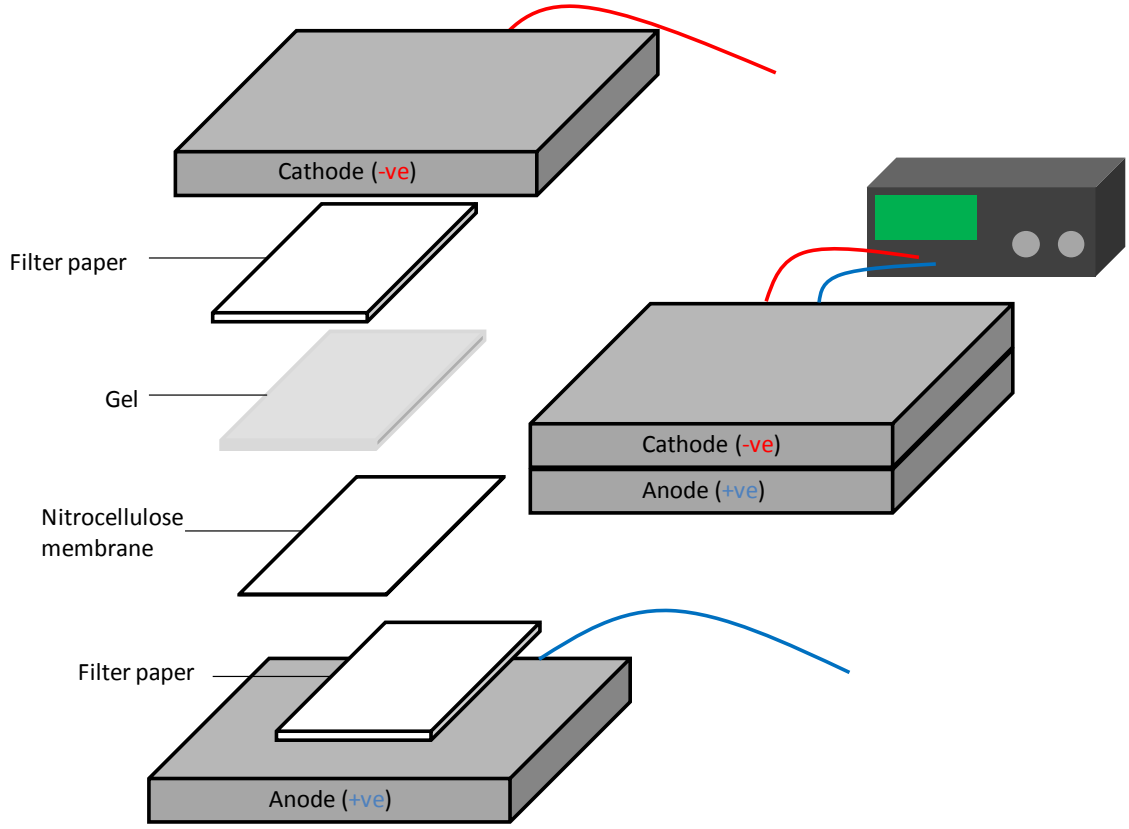


Figure 2.11: The different layers required in the transfer of proteins from a gel to a nitrocellulose membrane. The gel and nitrocellulose membrane are 'sandwiched' between 2 pieces of filter paper and placed in between electrodes. Electrotransfer (at 100mA) for 60min was required to fully transfer the proteins from the gel onto the nitrocellulose membrane.

2.7.6 Detection

To detect proteins of interest specific antibodies were added after the membrane had been blocked (required to prevent non-specific background binding of the primary

and/or secondary antibodies to the membrane, which has a high capacity at binding proteins and therefore antibodies). To prevent non-specific binding each membrane was blocked for 1h at room temperature with gentle agitation with 5% non-fat milk (Marvel®, Dublin, Republic of Ireland) in Tween-20 buffer (150mM NaCl, 10mM Tris-HCl pH 7.6 and 0.05 % (w/v) Tween-20) and then washed with Tween-20 buffer. The membranes were incubated overnight at 4°C with anti-XOR primary antibody (1:2000, Abcam, Cambridge, UK) diluted in 0.05% (w/v) Tween-20 buffer. The membranes were then incubated with polyclonal goat anti-mouse antibody (1:5000, Dako, Denmark) conjugated to horseradish peroxidase (HRP), diluted in Tween-20 buffer, for 1h at room temperature (figure 2.12).

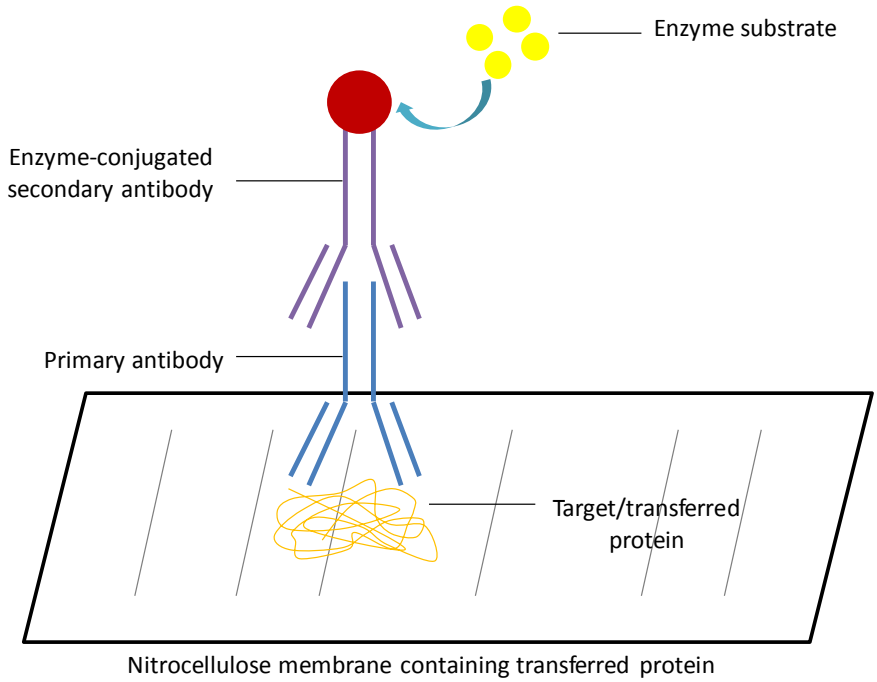


Figure 2.12: Detection of proteins using a primary antibody specific to the target protein of interest and a labelled secondary antibody.

In order to visualise the membrane the Clarity™ Western ECL kit was used (Bio-Rad, Hertfordshire, UK). Solution A (peroxide solution) and solution B (Luminol / enhancer solution) were mixed in a 1:1 ratio and the membrane incubated in the mixture for 2min. The excess was then drained and the membrane covered in Saran Wrap.

2.7.7 Data analysis

Densitometric analysis was performed on bands using the Gel Analyzer software (www.gelanalyzer.com) using the FluorChem™ E chemiluminescent western blot imaging system (ProteinSimple, Santa Clara, CA, USA). The levels of protein were expressed relative to β-actin expression.

2.8 Murine peritonitis

2.8.1 Induction of inflammation

Mice were injected intraperitoneally (i.p.) with 0.5ml of a pro-inflammatory stimulus of IL-1β (5ng), TNFα (300ng) or Zymosan (1mg). These doses were chosen as they have been shown to induce polymorphonuclear (PMN) leukocyte and monocyte accumulation into a site of acute inflammation (Perretti *et al.*, 1993; Dangerfield *et al.*, 2005; Cash *et al.*, 2009). Sterile PBS was used as a control. After 4 or 24h post injection the mice were anaesthetized using isoflurane (2%, vapourised in 100% oxygen at 0.4l/min, Baxter, UK). Blood was extracted by cardiac puncture prepared as described in section 2.3.1 for NO_x analysis. For the collection of peritoneal lavage fluid a small incision was made around the lower abdomen and the skin pulled apart to expose the underneath muscle without compromising the integrity of the peritoneal cavity. Using a 23-gauge needle 4ml of sterile ice cold PBS was injected in to the cavity. The cavity was thoroughly massaged for a minute and the wash retrieved using the same needle. The volume of wash was recorded and kept on ice. Tissue collection was conducted as described previously (see section 2.3.1).

2.8.2 Cell counting

Total white blood cell (WBC) count of the collected lavage fluid was assessed by dilution of sample with Turks solution (Merck, Germany). Turks solution haemolyses RBCs and stains the nuclei of WBCs blue to enable counting of leukocytes only. The total count was determined using a Neubauer haemocytometer (Hawksley, Sussex, UK) as recommended by the manufacturer using a x10 objective lens on the microscope (Eclipse TS100, Nikon, Surrey, UK).

2.8.3 Preparation for flow cytometry analysis

The lavage fluid was centrifuged for 5min, 4°C at 800g. The supernatant was then aliquoted for biochemical analysis and the cell pellet resuspended in a volume of FACS buffer (0.2% BSA in PBS) to yield 5 x 10^6cells/ml. A 100µl aliquot of each sample was added to FACS tubes and then centrifuged at 13,000g for 1 min at 4°C and the supernatant discarded. To block non-specific binding 20µl of Fc receptor antibody (1µg) was added to the cell pellets, vortexed to resuspend and incubated at 4°C for 15min. To each tube 10µl of the appropriate antibody was then added and the samples incubated in the dark at 4°C for 30 min. The cells were washed with 500µl of PBS then flow cytometry was performed to identify the cells. To exclude dead cells 5µl of the viability marker 7-AAD was added to all samples.

2.8.4 Flow cytometry

Flow cytometry is a technique that simultaneously measures multiple physical characteristics of single cells/particles as they flow in single file in a fluid stream (figure 2.13). The technique enables the user to gather detailed quantitative information on cells in a large population. After passing through a beam of light data such as relative size, relative granularity/internal complexity and relative fluorescence intensity, about a particle's properties are collated. These properties are determined using an optical-to-electron coupling system that records how the particle scatters incident laser light and emits fluorescence (figure 2.14). Samples were acquired and recorded on the LSRFortessa II Cell Analyzer (BD Biosciences, San Jose, CA, USA) using BD FACSDiva Software (BD Biosciences, San Jose, CA, USA).

The flow cytometer analysed deflected light from each cell, which is recorded as forward-scatter (FSC; proportional to the size of the cell) and side-scatter (SSC; relative to granularity and internal complexity of the cell) on a FSC/SSC dot-plot graph (Figure 2.15). Fluorochromes conjugated to antibodies can bind to specific cell surface markers and the presence of these markers can be quantified by measuring the fluorescence emission.

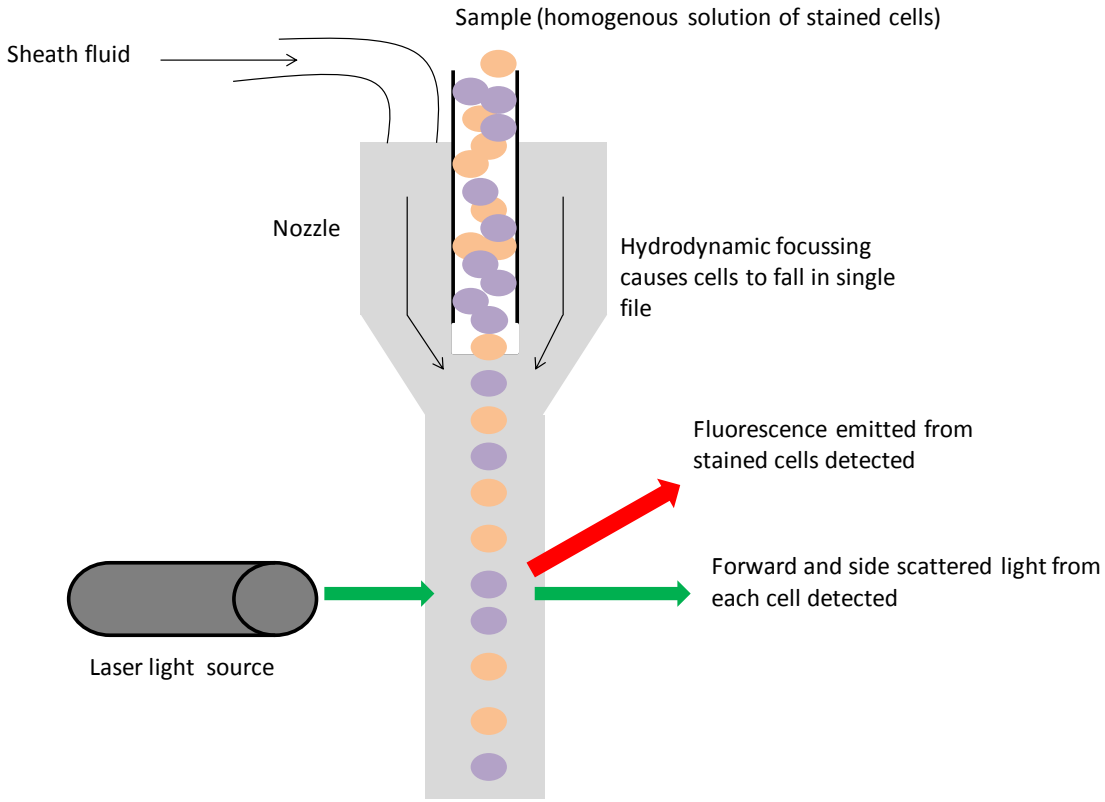


Figure 2.13: Diagram of the basic internal structure of a flow cytometer. Sheath fluid passing down the nozzle aids the cells to fall in single file allowing the acquisition of data for each individual cell to be done.

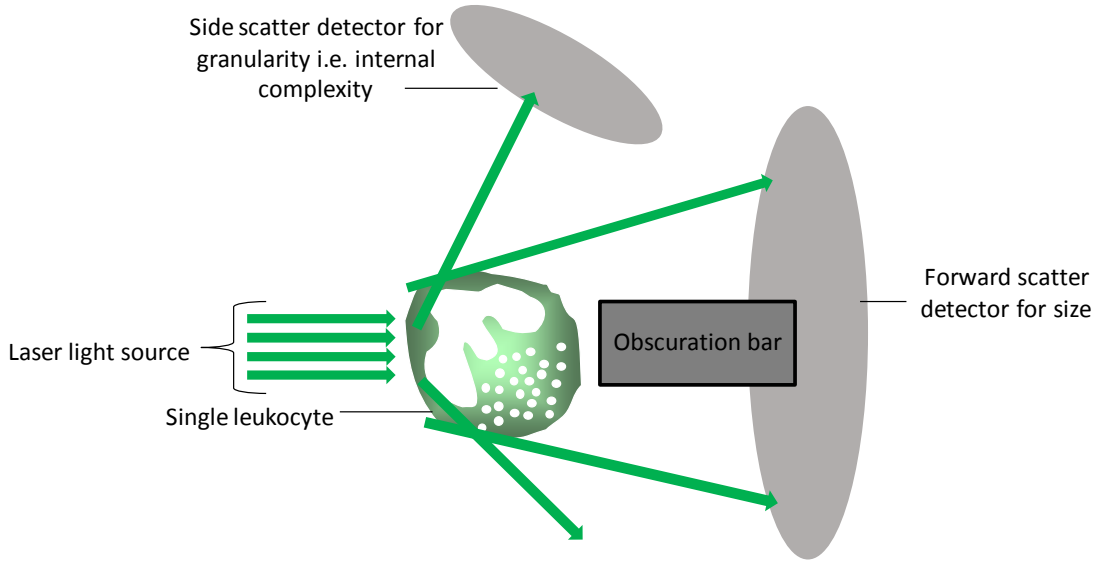


Figure 2.14: Determination of cell specific properties by an optical-to-electron coupling system. Forward and side scatter detection means information about the size and internal complexity of a cell is acquired.

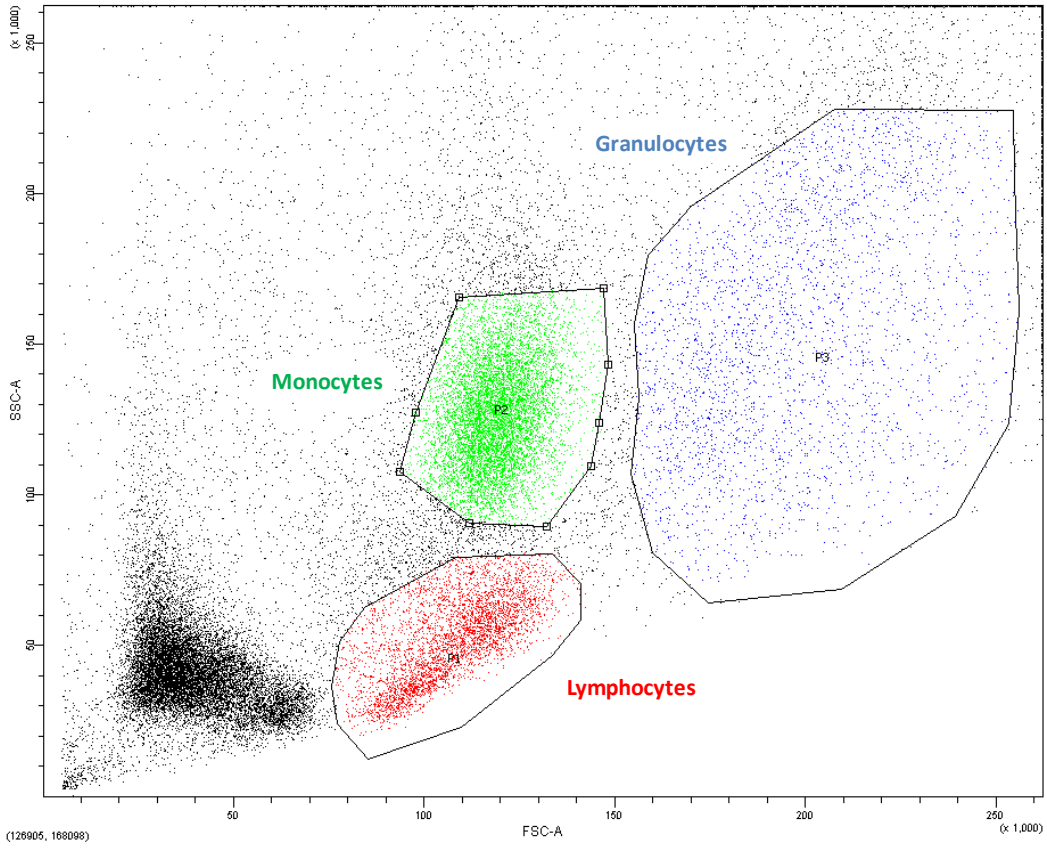


Figure 2.15: Dot plot showing the FSC vs. SSC characteristics of leukocyte subpopulations derived from mouse lavage fluid. Lymphocytes are the smallest leukocytes with little granularity in the cytoplasm. They are identified by having a low FSC and SSC (P1 gate; red). Monocytes (P2 gate; green) are bigger and therefore distinguished by a larger FSC. Granulocytes (predominantly neutrophils) are the largest cell type and contain many granules within their cytoplasm and therefore have a larger FSC and SSC (P3 gate; blue). The black dots with low FSC and SSC represent dead cells and/or debris.

The different leukocyte subtypes express different patterns of markers that enable characterization, and were further identified by their distinct expression profiles of F4/80 and Gr1 i.e. resident monocytes ($F4/80^+$, $Gr1^-$), inflammatory monocytes ($F4/80^+$, $Gr1^+$) and neutrophils ($F4/80^-$, $Gr1^+$). Furthermore the expression of the inflammatory markers CD62L, CD162 and CD11b on the surfaces of these cells was investigated. Tables 2.7 and 2.8 give a more detailed account of the information elucidated from using these particular markers.

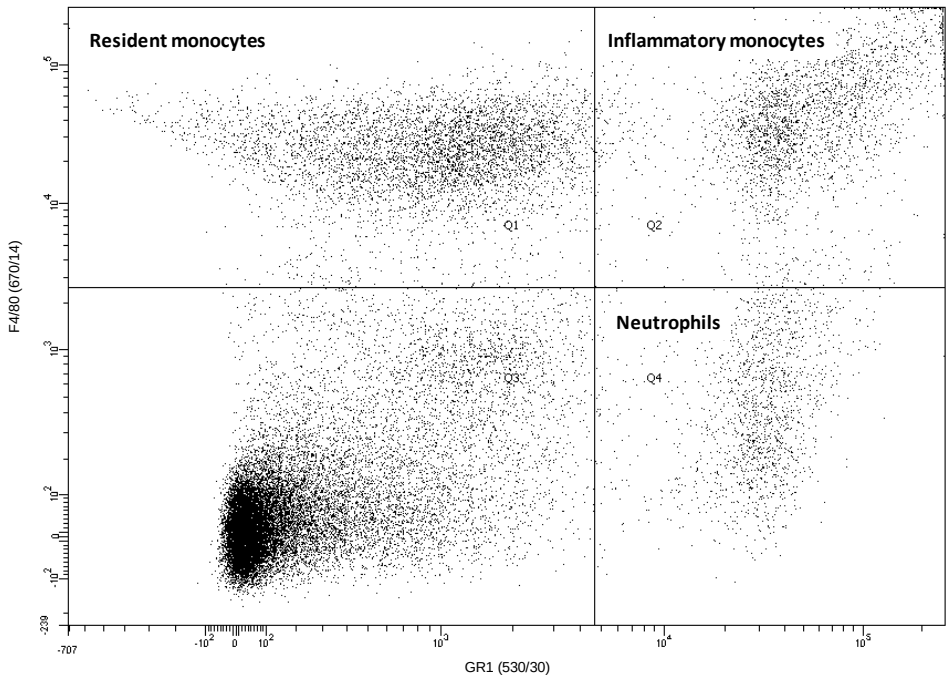


Figure 2.16: Quadrant scheme for the identification of different leukocyte subsets. Subsets include Q1 resident monocytes ($F4/80^{+}$, $Gr1^{-}$), Q2 inflammatory monocytes ($F4/80^{+}$, $Gr1^{+}$) and Q4 neutrophils ($F4/80^{-}$, $Gr1^{+}$).

Antigen	Cell Type	Fluorochrome Conjugated With Antibody
Gr1	neutrophils	Fluorescein isothiocyanate (FITC)
F4/80	resident monocytes	Allophycocyanin (APC)
GR1 + F4/80	inflammatory monocytes	

Table 2.7: The fluorochrome conjugated antibodies used to identify specific inflammatory cells.

Antigen	Alternative Name	Role Of Surface Marker	Fluorochrome Conjugated With Antibody
CD62L	L-selectin	Expressed by activated macrophages, monocytes and neutrophils. Important for leukocyte rolling	APC-eFluor 780
CD162	P-selectin glycoprotein ligand-1 (PSGL-1)	Expressed by activated leukocytes, PSGL-1 is the high affinity receptor for P-selectin (expressed by platelets and endothelial cells, important for leukocyte rolling)	Phycoerythrin (PE)
CD11b	Integrin alpha-M beta-2 (αMβ2)	Expressed by activated leukocytes. Aids in leukocyte adhesion and migration	eFluor 450

Table 2.8: The fluorochrome conjugated antibody used to identify specific cell surface markers.

Combined with FSC and SSC data the staining pattern of each leukocyte was used to calculate the relative percentage of cells expressing each inflammatory marker, as well as their respective median fluorescence intensity (MFI). MFI is a measure of the number of markers present on each cell. By isolating sub-populations of leukocytes, it was possible to determine what percentage of total leukocytes was made up of each inflammatory cell type. To ensure that the fluorescence recorded was due to specific primary antibody binding to the desired marker, and not due to non specific binding (i.e. Fc receptors), isotype-matched control antibodies were used. Figure 2.17 shows an example of the histograms obtained from the antibody marker and its respective isotype control.

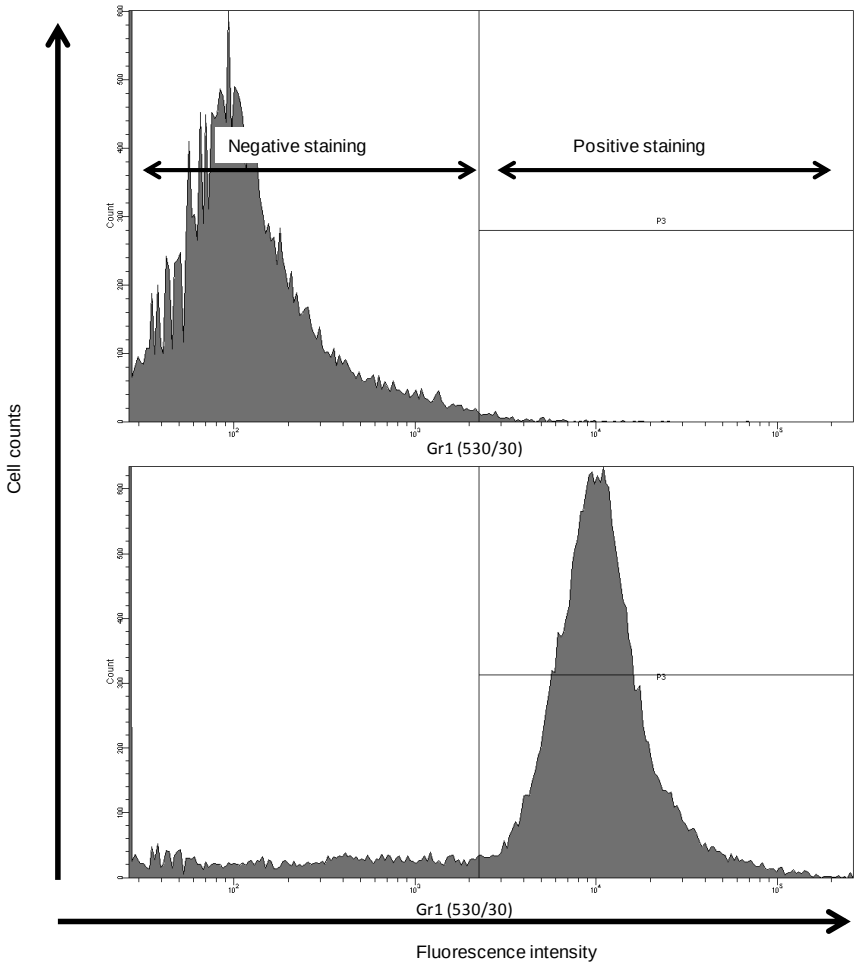


Figure 2.17: An example histogram of cells stained positively for Gr1 and the isotype control for Gr1. The top graph shows an example histogram of cells positively stained for Gr1. The bottom graph shows and example of cells positively stained for the isotype control for Gr1. This analysis was set up individually for each antibody.

Flow cytometry data were analysed using the BD FACSDiva 6.0 software. During flow cytometry the fluorochromes are excited by light which causes the emission of fluorescent light at a longer wavelength. The fluorochromes do not emit light at a specific wavelength but over a fairly wide range known as emission spectra. Overlapping of emission spectra between fluorochromes can occur therefore when using multiple fluorochromes, the emitted light from the leading and trailing tails from a neighbouring fluorochrome is registered. Since 5 different fluorochromes were used spectral overlap had to be accounted for by manual compensation to allow accurate data analysis. In order to manually compensate, single staining of samples with each antibody conjugated fluorochrome was recorded on the flow cytometer.

2.8.5 Data analysis

Analysis of cell population size and fluorescent intensities of the different leukocytes were achieved by constructing dot plots and histograms. In order to segregate leukocyte populations accurately, gates had to be set-up on the dot-plots. The quadrant boundaries between cells that stained positive and negative for a particular leukocyte population was determined according to the fluorescence distribution of positively stained cell relative to the unstained sample.

2.9 Myeloperoxidase assay

Myeloperoxidase (MPO) is a protein abundantly expressed by neutrophils and produces hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) and the chloride anion (Cl^-) during the neutrophils respiratory burst. Measurement of MPO is standardly taken as a good indicator of neutrophil recruitment to a particular site of interest. This assay was used to confirm whether neutrophils were recruited in response to i.p. administration of inflammogens.

Mesentery samples from PBS, zymosan, TNFα or IL-1β treated mice were homogenised in 1 ml of 0.5% hexadecyltrimethylammonium bromide (HTAB) using a precellys homogeniser (Bertin Technologies, France) and CK14 beaded tubes (Stretton Scientific, Derbyshire, UK). Samples were centrifuged 2 times at 5,000rpm for 30s with 15s between centrifugations. Following homogenisation samples were then centrifuged at 10,000g for 5min at 4°C to generate a supernatant, which was then collected and the pellet discarded. A standard curve was generated using purified MPO in PBS (2U/ml) by serial dilution at 0.03125-1U/ml). Standard and sample (20µl) were loaded in triplicate on a 96-well plate and 160µl of reagent (Tetramethylbenzidine (TMB) 4mg/ml in DMSO then diluted 1:8 in PBS just before use) was added to each of the wells along with 20µl of H_2O_2 (0.1mM diluted 1:30 in PBS just before use). The plate was protected from light using tin foil and left to incubate for 5min. Light absorbance was measured using a spectrophotometric plate reader (MRX-TC Revelation, Dynex Technologies, UK) at 620nm and sample MPO concentration determined from the

standard curve. In addition, as previously described (section 2.4.3) a protein assay was conducted to determine the protein concentration of each supernatant sample.

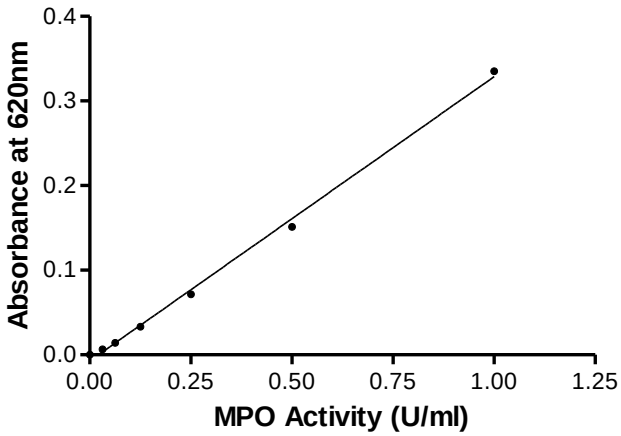


Figure 2.18: Typical standard curve generated from the MPO assay.

2.9.1 Data analysis

To normalize data to enable comparison between treatments, all data were corrected for protein amount therefore the final units of MPO activity were expressed as U per mg.

2.10 Statistical analysis

All data were analysed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). All values are expressed as mean±standard error of the mean (SEM) with the *n* value representing the number of animals or patient in that particular treatment group unless otherwise stated. A statistically significant result was indicated by a probability level, p, of less than 0.05.

For the analysis in WT mice 1-way analysis of variance (ANOVA) was used to compare means as the means of more than 2 unmatched treatment groups was being tested. A Dunnett's multiple comparison post-test was applied to compare the mean of each treatment group with the control mean. When examining the effect that atherosclerosis had on NO_x distribution a 2-way ANOVA was used as the mean differences between groups split on 2 independent variables (in this case genotype and dietary treatment) was being examined. The Bonferroni post-test was applied.

Unpaired students t-tests were used for comparisons between baseline statistics as samples were collected from different animals. A 2-tailed test was used as it was not clear as to how means would differ.

For the assessment of nitrite reductase activity, when comparing concentration-response curves between health and disease (i.e. WT vs. ApoE KO or WKY vs. SHR) or in the absence or presence of enzyme inhibitors, statistical significance was established with 2-way ANOVA followed by Bonferroni's post-tests.

For the peritonitis studies 1-way ANOVA with Dunnett's multiple comparison test was used for comparison of the 2 nitrate treatments with baseline control.

Chapter 3:

Investigation of the distribution of nitrite and nitrate in models of cardiovascular disease

3.1 Introduction

CVD is associated with a deficiency of NO_x therefore strategies that aim to restore levels might have therapeutic utility. Previous studies have demonstrated that an approximate doubling of circulating nitrite levels is associated with significant biological activity. In healthy volunteers, a 24mmol dose of inorganic nitrate (given in 500ml of beetroot juice) caused a 2-fold elevation in plasma nitrite concentration (Webb *et al.*, 2008c). This elevation in plasma nitrite levels was paralleled with significant reductions in blood pressure and improvement in endothelial function (Webb *et al.*, 2008c). In a similar vein inorganic nitrate supplementation in the form of potassium nitrate capsules also exerted blood pressure lowering and endothelial effects (Kapil *et al.*, 2010) in a dose-dependent manner associated with dose-dependent increases in plasma nitrate and nitrite concentration (Kapil *et al.*, 2010). However, whether similar elevation of both anions might occur in tissues and whether the distribution of these anions at baseline and following supplementation might be altered in CVD has not been systematically investigated.

The aim of this study was to:

1. Determine whether nitrite and/or nitrate levels are altered in the cardiovascular system between health and disease.
2. Determine whether nitrite or nitrate might be used effectively to elevate NO_x levels throughout the cardiovascular system in both health and CVD.

3.2 Assessment of the effect of nitrite and nitrate supplementation on basic parameters in WT mice

3.2.1 Testing the stability of the drinking water

Before administering nitrite/nitrate through the drinking water it was first necessary to determine the stability of the anions in the water over time. In all cases the concentration of either the nitrate or nitrite ion remained constant for no more than 2 days (figure 3.1) before declining and becoming significantly reduced at 8 days.

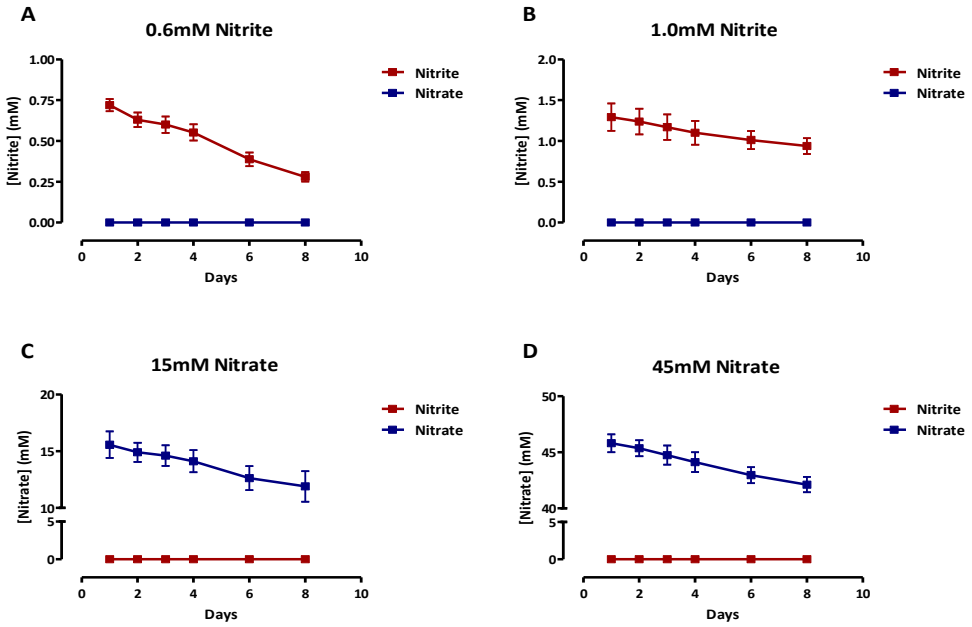


Figure 3.1: The effect of time on the stability of either nitrite or nitrate anion in the drinking water. Data are expressed as mean±SEM (0.6mM nitrite n=4; 1.0mM nitrite n=4; 15mM nitrate n=4; 45mM nitrate n=4).

Based on these initial studies it was decided that the drinking water would be made up fresh and replaced every 2 days.

3.2.2 Food and water consumption

Nitrite or nitrate supplementation appeared to have no impact on food (figure 3.2A) or water consumption (figure 3.2B). An estimate of the amount of nitrite or nitrate consumed per day demonstrated that the doses used were equivalent to 7.00 and 13.56mg/kg/day nitrite and 7.59, 207.00 and 698.90mg/kg/day nitrate (table 3.1).

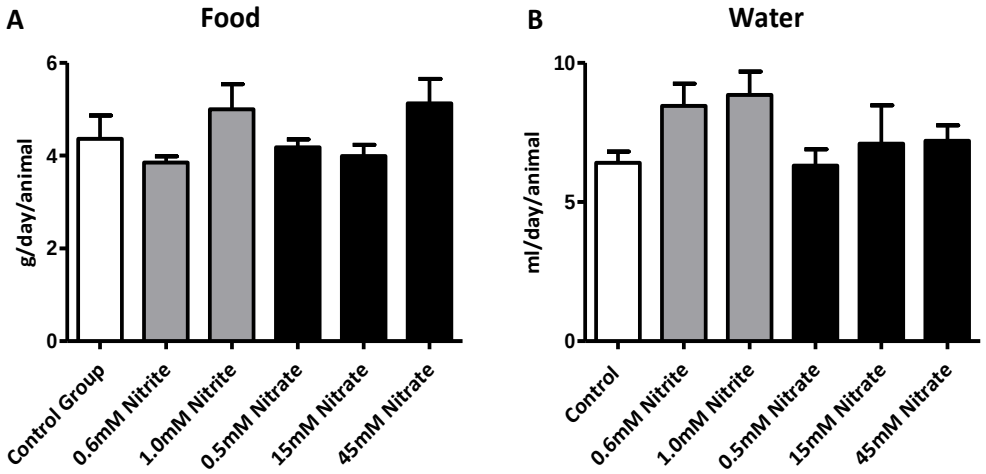


Figure 3.2: The effect of drug treatment on food (A) and water (B) consumption. Data are expressed as the average amount consumed per day per animal. Data are expressed as mean±SEM (control group n=11; 0.6mM nitrite n=12; 1.0mM nitrite n=8; 0.5mM nitrate n=8; 15mM nitrate n=9; 45 mM nitrate n=9). Data analysed using a one-way ANOVA Dunnett's multiple comparison statistical test.

		Amount nitrite consumed		**Amount nitrate consumed**	
Treatment	**Water consumed (ml/day)**	**(mg/kg/day)**	**(mmol/kg/day)**	**(mg/kg/day)**	**(mmol/kg/day)**
Control	6.41±0.41	<0.001	<0.001	<0.001	<0.001
0.6mM Nitrite	8.46±0.80	7.00±0.20	0.15±0.01	<0.001	<0.001
1.0mM Nitrite	8.85±0.84	13.56±0.43	0.29±0.01	<0.001	<0.001
0.5mM Nitrate	6.31±0.59	<0.001	<0.001	7.59±0.35	0.12±0.01
15mM Nitrate	7.11±1.36	<0.001	<0.001	207.00±11.25	3.34±0.18
45mM Nitrate	7.20±0.55	<0.001	<0.001	698.90±38.73	11.27±0.62

Table 3.1: The amount of nitrite and nitrate consumed by WT mice during the 2 week treatment period. Values are expressed as mean±SEM.

3.2.3 Weight gain over time

Neither nitrite nor nitrate affected weight gain during the treatment period (figure 3.3, table 3.2).

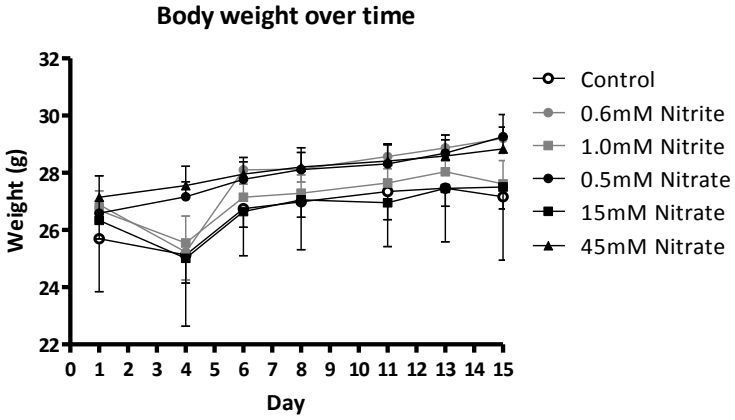


Figure 3.3: The effect of nitrite/nitrate supplementation on weight gain throughout the 2 week feeding period. Data are expressed as the average mean±SEM (control group n=11; 0.6mM nitrite n=12; 1.0mM nitrite n=8; 0.5mM nitrate n=8; 15mM nitrate n=9; 45 mM nitrate n=9).

Treatment	n value	Weight prior to study (g)	Weight at the end of the study (g)	Overall weight change (g)
Control	11	25.7±0.51	27.7±0.83	2.0±0.32
0.6mM nitrite	12	26.9±0.54	28.2±0.79	1.4±0.25
1.0mM nitrite	8	26.7±0.69	27.6±0.82	0.9±0.13
0.5mM nitrate	8	26.6±0.43	29.3±0.78	2.7±0.35
15mM nitrate	12	26.3±0.65	27.5±0.76	1.2±0.12
45mM nitrate	8	27.1±0.75	28.5±0.81	1.4±0.06

Table 3.2: Summary of the average weight of the WT mice prior to and at the end of the study. Data are expressed as mean±SEM.

3.3 Effect of nitrite/nitrate supplementation on plasma NO_x in WT mice

In WT mice nitrite supplementation appeared to slightly elevate plasma nitrite but not plasma nitrate concentration (figure 3.4A + 3.4B). Nitrate treatment caused a concentration dependent rise in both plasma nitrite and nitrate concentration (figure 3.4A + 3.4B), which reached significance at the 15 and 45mM concentrations.

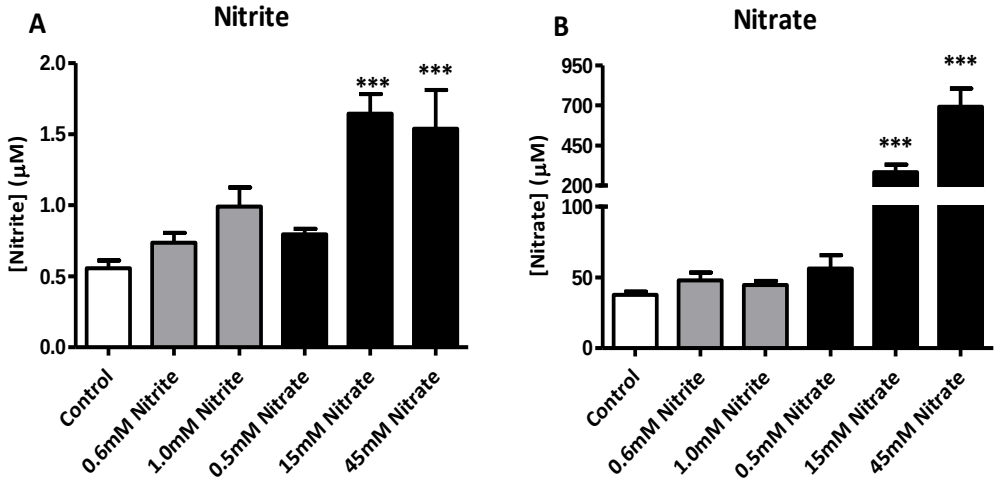


Figure 3.4: The effect of dietary nitrite and dietary nitrate supplementation on plasma nitrite (A) and nitrate (B) concentration in WT mice. Data are expressed as mean ± SEM (control group n=11; 0.6mM nitrite n=12; 1.0mM nitrite n=8; 0.5mM nitrate n=8; 15mM nitrate n=12; 45mM nitrate n=8). A significant difference is shown as ***p<0.001 vs. control group using a one-way ANOVA Dunnett's multiple comparison statistical test.

From these studies it was decided that all further investigations in mice would be conducted with only a single dose of each anion for supplementation i.e. 0.6mM nitrite and 15mM nitrate. These doses were chosen since they caused rises in circulating nitrite that was commensurate with rises associated with biological activity in healthy volunteers (Kapil *et al.*, 2010).

3.4 Comparison of baseline NO_x levels between WT vs. ApoE KO mice in the blood

There was no statistical difference in baseline plasma and RBC nitrite levels between WT and ApoE KO mice however, there was a significant reduction in nitrate levels (table 3.3).

	Nitrite			Nitrate		
	WT	**ApoE KO**	**p value**	**WT**	**ApoE KO**	**p value**
Plasma (μM)	0.56±0.10 (n=11)	0.45±0.06 (n=12)	0.18	37.68±2.26 (n=11)	19.99±2.80 (n=12)	<0.0001
RBC (nmoles/g protein)	0.41±0.05 (n=5)	0.33±0.05 (n=5)	0.26	13.06±1.40 (n=5)	6.33±1.34 (n=5)	<0.01

Table 3.3: Comparison of baseline levels of nitrite and nitrate in plasma and blood. Statistical comparison using unpaired t-test.

3.4.1 The effects of dietary supplementation on distribution of NO_x in the blood

Nitrite supplementation caused slight elevations in plasma nitrite levels for both genotypes but these were not significant, however nitrate supplementation significantly elevated plasma nitrite levels (figure 3.5A). In addition a dietary nitrite strategy appeared to have no effect on nitrate levels, however dietary nitrate supplementation significantly elevated nitrate levels in both WT and ApoE KO mice (figure 3.5B).

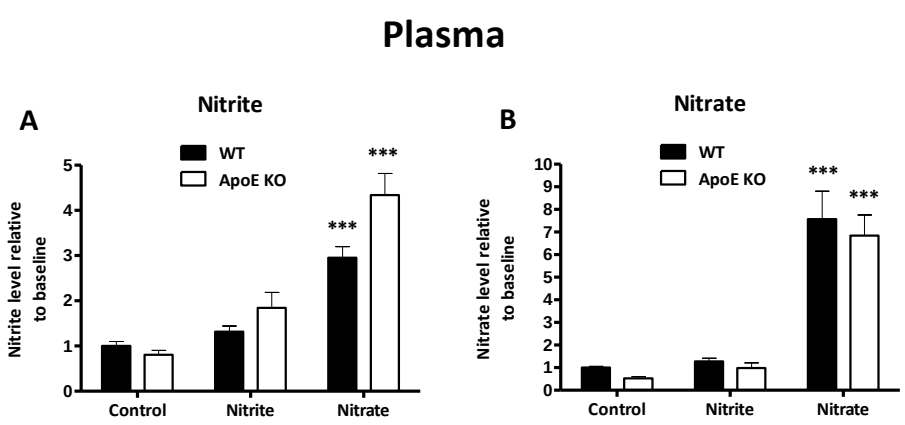


Figure 3.5: The effect of dietary nitrite and dietary nitrate supplementation on plasma nitrite (A) and nitrate (B) levels in WT vs. ApoE KO mice (WT control n=11; 0.6mM nitrite n=12; 15mM nitrate n=12, ApoE KO control n=12; 0.6mM nitrite n=11; 15mM nitrate n=11). Data shown as mean±SEM of levels normalized to baseline. A significant difference is show as ***p<0.001 vs. control group using a 1-way ANOVA Dunnett's multiple comparison statistical test.

Dietary nitrite and nitrate supplementation significantly elevated nitrite levels for WT and ApoE KO mice (figure 3.6A). Furthermore dietary nitrite supplementation appeared to have no effect on nitrate levels, however dietary nitrate supplementation did cause significant elevations of nitrate levels for both genotypes (figure 3.6B).

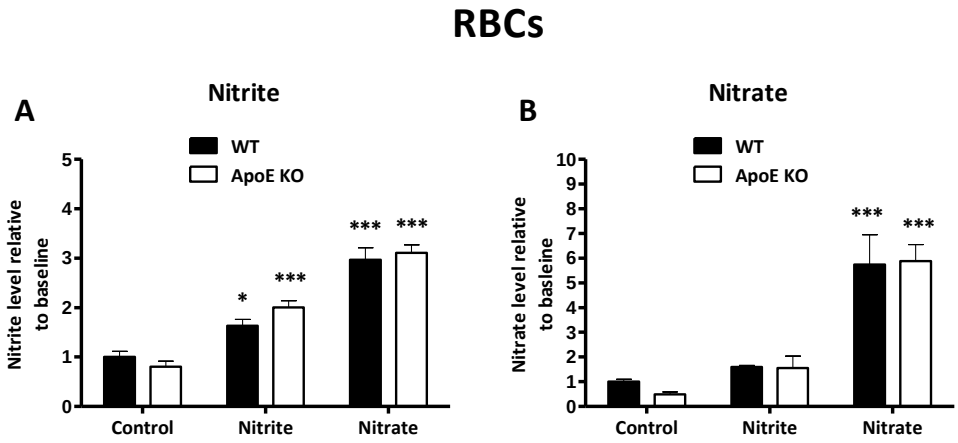


Figure 3.6: The effect of dietary nitrite and dietary nitrate supplementation on RBC nitrite (A) and nitrate (B) levels in WT vs. ApoE KO mice (WT control n=5; 0.6mM nitrite n=5; 15mM nitrate n=5, ApoE KO control n=5; 0.6mM nitrite n=5; 15mM nitrate n=5). Data shown as mean±SEM of levels normalized to baseline. A significant difference is show as ***p<0.001 and **p<0.01 vs. control group using a 1-way ANOVA Dunnett's multiple comparison statistical test.

3.5 Comparison of baseline NO_x levels between WT vs. ApoE KO mice in the tissues

There was no statistical difference between the genotypes in baseline tissue nitrite levels, however baseline tissue nitrate levels were significantly reduced in ApoE KO vs. WT mice (table 3.4). This was applicable for all tissues apart from the mesentery in which nitrite levels were significantly raised in the ApoE KO and nitrate levels were also raised in the ApoE KO although this did not reach significance. Interestingly, for both nitrite and nitrate the aorta had the highest levels, whilst the rest of the tissues had similar levels of nitrite and nitrate.

	Nitrite (nmoles/g protein)			**Nitrate (nmoles/g protein)**		
	WT	**ApoE KO**	**p value**	**WT**	**ApoE KO**	**p value**
Aorta	102.51±7.28 (n=10)	112.85±10.11 (n=13)	0.44	1014.44±142.53 (n=10)	503.73±51.60 (n=13)	<0.01
Kidney	4.93±0.66 (n=13)	5.19±0.73 (n=10)	0.80	352.58±36.89 (n=13)	105.11±13.80 (n=10)	<0.0001
Lung	12.57±1.43 (n=11)	17.38±1.82 (n=11)	0.05	349.05±33.33 (n=11)	148.37±31.94 (n=11)	<0.001
Heart	4.73±0.67 (n=11)	3.29±0.58 (n=11)	0.12	204.85±13.27 (n=11)	126.86±12.07 (n=11)	<0.001
Liver	5.01±0.65 (n=13)	4.37±0.67 (n=12)	0.50	130.74±12.76 (n=13)	80.32±14.69 (n=12)	<0.05
Mesentery	29.24±1.89 (n=11)	55.96±5.16 (n=8)	<0.0001	287.13±37.16 (n=11)	426.14±61.11 (n=8)	0.056

Table 3.4: Comparison of baseline levels of nitrite and nitrate in tissue homogenates. Statistical comparison using unpaired t-test between WT and ApoE KO mice.

3.5.1 The effects of dietary supplementation on distribution of NO_x in the tissues

Aorta nitrite levels were not elevated by dietary nitrite supplementation but were significantly elevated by dietary nitrate supplementation for both genotypes (figure 3.7A). As with nitrite, dietary nitrite appeared to have no effect on nitrate levels but nitrate supplementation significantly elevated nitrate levels for both genotypes (figure 3.7B).

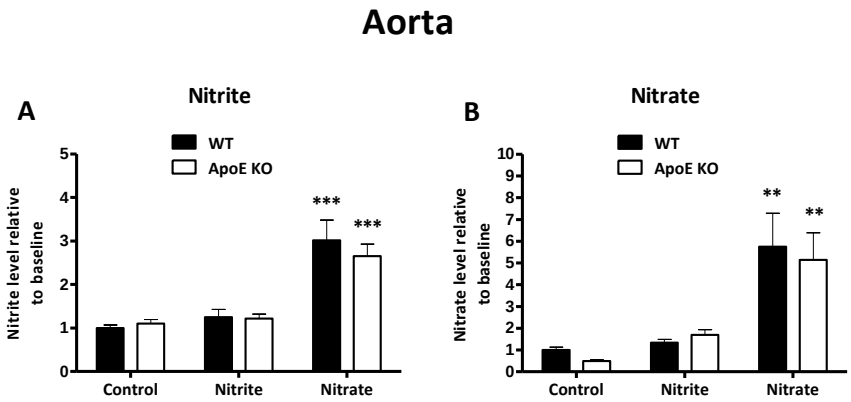


Figure 3.7: The effect of dietary nitrite and dietary nitrate supplementation on aorta nitrite (A) and nitrate (B) levels in WT vs. ApoE KO mice (WT control n=10; 0.6mM nitrite n=10; 15mM nitrate n=10, ApoE KO control n=13; 0.6mM nitrite n=13; 15mM nitrate n=13). Data shown as mean±SEM of levels normalized to baseline. A significant difference is show as ***p<0.001 and **p<0.01 vs. control group using a 1-way ANOVA Dunnett's multiple comparison statistical test.

Kidney nitrite levels were not elevated by a dietary nitrite strategy but were significantly elevated by a dietary nitrate strategy for both genotypes (figure 3.8A). This was reflected in nitrate levels since dietary nitrite supplementation had no effect, however dietary nitrate supplementation significantly elevated nitrate levels for both genotypes (figure 3.8B).

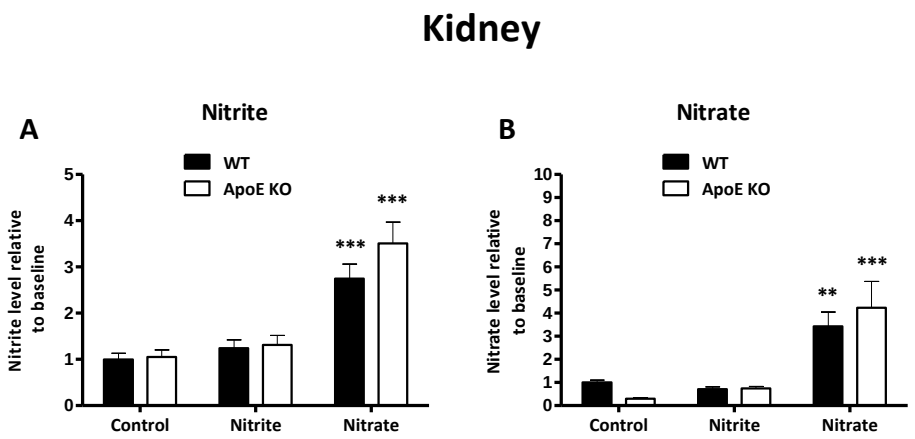


Figure 3.8: The effect of dietary nitrite and dietary nitrate supplementation on kidney nitrite (A) and nitrate (B) levels in WT vs. ApoE KO mice (WT control n=13; 0.6mM nitrite n=10; 15mM nitrate n=10, ApoE KO control n=10; 0.6mM nitrite n=10; 15mM nitrate n=9). Data shown as mean±SEM of levels normalized to baseline. A significant difference is show as ***p<0.001 and **p<0.01 vs. control group using a 1-way ANOVA Dunnett's multiple comparison statistical test.

For lung homogenates nitrite levels were significantly elevated by dietary nitrate supplementation but not dietary nitrite supplementation for both WT and ApoE KO mice (figure 3.9A). In addition, nitrate levels were significantly elevated by dietary nitrate supplementation but not by dietary nitrite supplementation for both genotypes (figure 3.9B).

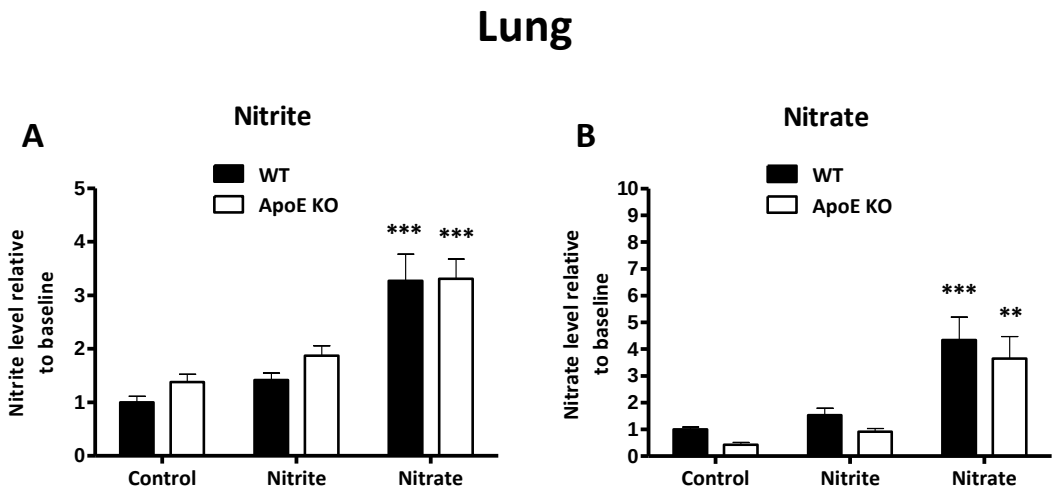


Figure 3.9: The effect of dietary nitrite and dietary nitrate supplementation on lung nitrite (A) and nitrate (B) levels in WT vs. ApoE KO mice (WT control n=11; 0.6mM nitrite n=11; 15mM nitrate n=11, ApoE KO control n=11; 0.6mM nitrite n=11; 15mM nitrate n=11). Data shown as mean±SEM of levels normalized to baseline. A significant difference is show as ***p<0.001 and **p<0.01 vs. control group using a 1-way ANOVA Dunnett's multiple comparison statistical test.

Statistically significant elevations of nitrite levels in heart homogenates were observed with both WT and ApoE KO mice that had been treated with dietary nitrate supplementation but not dietary nitrite supplementation (figure 3.10A). This was reflected in nitrate levels as well since dietary nitrate treatment caused statistically significant elevations of nitrate levels for both genotypes whereas dietary nitrite treatment had no effect (figure 3.10B).

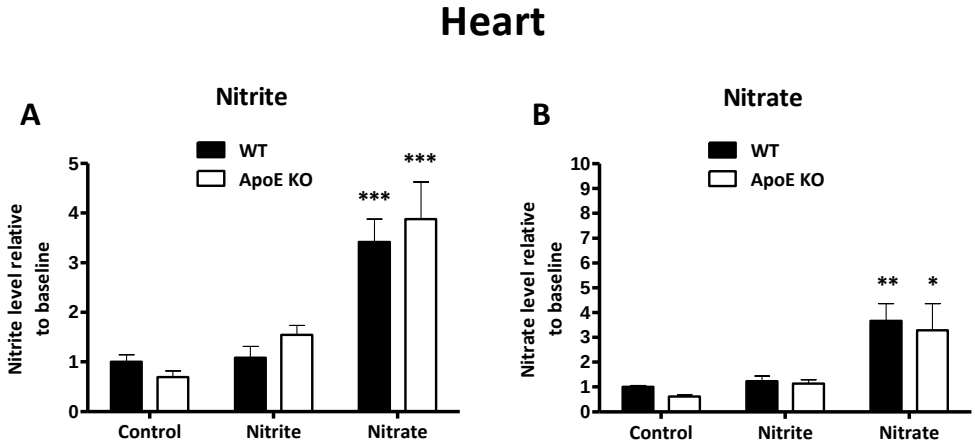


Figure 3.10: The effect of dietary nitrite and dietary nitrate supplementation on heart nitrite (A) and nitrate (B) levels in WT vs. ApoE KO mice (WT control n=11; 0.6mM nitrite n=10; 15mM nitrate n=10, ApoE KO control n=11; 0.6mM nitrite n=11; 15mM nitrate n=11). Data shown as mean±SEM of levels normalized to baseline. A significant difference is show as ***p<0.001 vs. control group using a 1-way ANOVA Dunnett's multiple comparison statistical test.

In liver homogenates, dietary nitrite supplementation had no effect on nitrite levels for both genotypes whereas dietary nitrate supplementation significantly elevated nitrite levels (figure 3.11A). Furthermore, dietary nitrite supplementation had no effect on nitrate levels but dietary nitrate supplementation significantly elevated nitrate levels for both WT and ApoE KO mice (figure 3.11B).

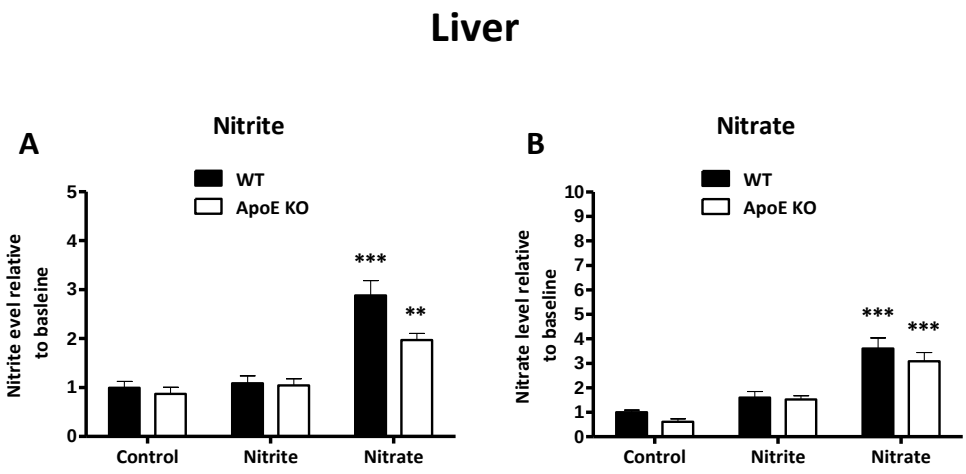


Figure 3.11: The effect of dietary nitrite and dietary nitrate supplementation on liver nitrite (A) and nitrate (B) levels in WT vs. ApoE KO mice (WT control n=13; 0.6mM nitrite n=13; 15mM nitrate n=13, ApoE KO control n=12; 0.6mM nitrite n=12; 15mM nitrate n=12). Data shown as mean±SEM of levels normalized to baseline. A significant difference is show as ***p<0.001 and **p<0.01 vs. control group using a 1-way ANOVA Dunnett's multiple comparison statistical test.

Dietary nitrite supplementation had no effect on nitrite levels in WT mice but significantly elevated nitrite levels in mesentery homogenates of ApoE KO mice. Furthermore dietary nitrate supplementation significantly elevated nitrite levels in both genotypes (figure 3.12A). Interestingly, dietary nitrite supplementation appeared to have no effect on nitrate levels but dietary nitrate supplementation significantly elevated nitrate levels for both WT and ApoE KO mice (figure 3.12B).

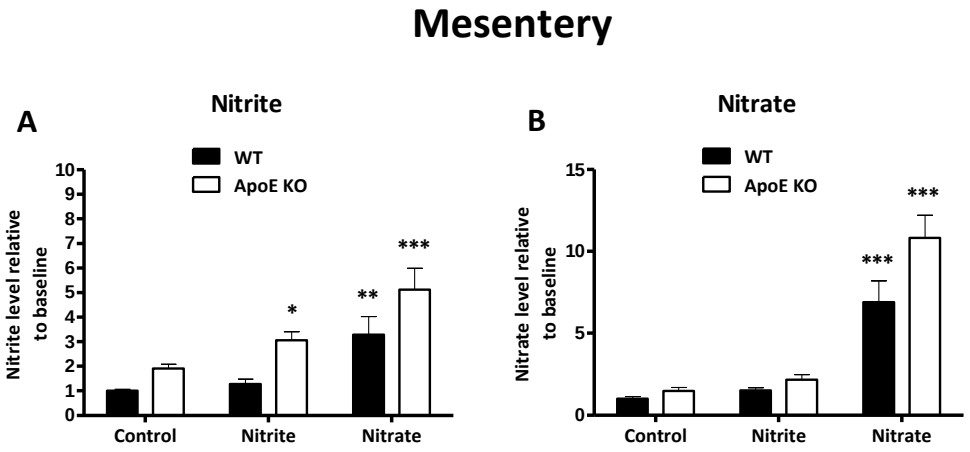


Figure 3.12: The effect of dietary nitrite and dietary nitrate supplementation on mesentery nitrite (A) and nitrate (B) levels in WT vs. ApoE KO mice (WT control n=11; 0.6mM nitrite n=11; 15mM nitrate n=11, ApoE KO control n=8; 0.6mM nitrite n=8; 15mM nitrate n=8). Data shown as mean±SEM of levels normalized to baseline. A significant difference is show as ***p<0.001, **p<0.01 and *p<0.05 vs. control group using a 1-way ANOVA Dunnett's multiple comparison statistical test.

3.6 Comparison of baseline NO_x levels between WKY vs. SHRs

The first animal model of hypertension was developed in 1934 by Harry Goldblatt (Goldblatt *et al.*, 1934). Rats are the most popular animal used to model hypertension with the spontaneously hypertensive rat (SHR) being the most often used (Pinto *et al.*, 1998). The SHR is characterized by progressively increasing arterial pressure, vascular dysfunction and eventual left ventricular hypertrophy (Sullivan *et al.*, 2010). For the first 5-6 weeks SHRs are pre to mildly hypertensive with conscious systolic blood pressure being 120-150mmHg (Touyz *et al.*, 1999) (spontaneous hypertension is characterized by blood pressure exceeding 150mmHg persistently over 4 weeks (Okamoto *et al.*, 1963)). SHRs show clear sex differences in their progression of hypertension (female SHRs develop hypertension slower, although by 24 months of age blood pressures are similar (Chan *et al.*, 2011)), therefore only males were used. At 16 weeks of age, SHRs compared to WKY strain controls were hypertensive (table 3.5). In these experiments a comparison between baseline levels across the compartments between WKY and SHRs was made. Due to cost and availability the impact of dietary nitrite or nitrate was not feasible.

Parameter	WKY (n=12)	SHR (n=16)	p value
SBP, mmHg	119.3±2.6	154.4±3.9***	<0.0001
DBP, mmHg	74.4±3.8	101.1±2.7***	<0.0001
MAP, mmHg	95.6±3.6	126.8±2.3***	<0.0001
HR, bpm	394.8±14.6	329.4±5.4***	<0.0001

Table 3.5: Baseline hemodynamic parameters in WKY rats and SHRs. Data shown as mean±SEM. Mean with statistical significance determined by 1-way ANOVA with ***p<0.0001 vs. WKY with Bonferroni post hoc analysis. Bpm indicates beats per min; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; SBP, systolic blood pressure.

3.6.1 Comparison of baseline NO_x levels in the blood

Plasma nitrite (figure 3.13A) and nitrate (figure 3.13B) concentration were similar in SHRs compared to WKY controls.

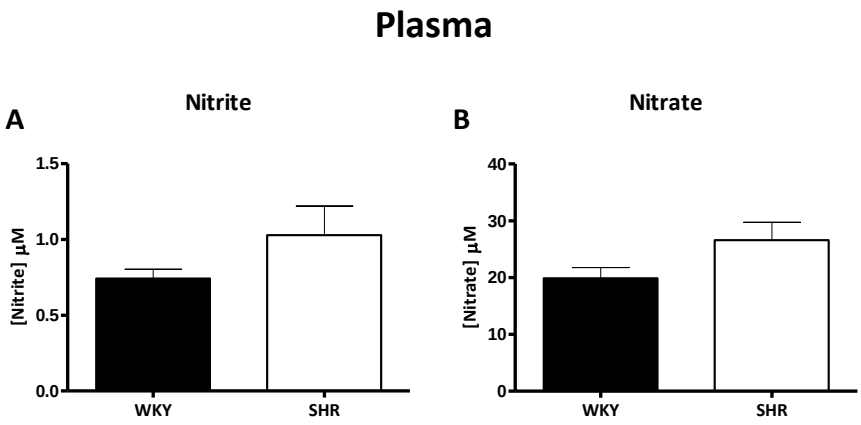


Figure 3.13: The concentration of nitrite (A) and nitrate (B) in the plasma of WKY (n=25) and SHRs (n=23). Data shown as mean±SEM. Comparison between 2 groups made using unpaired t-tests.

In contrast the levels of both nitrite (figure 3.14A) and nitrate (figure 3.14B) in purified RBCs were significantly reduced in the SHR compared to the WKY animals.

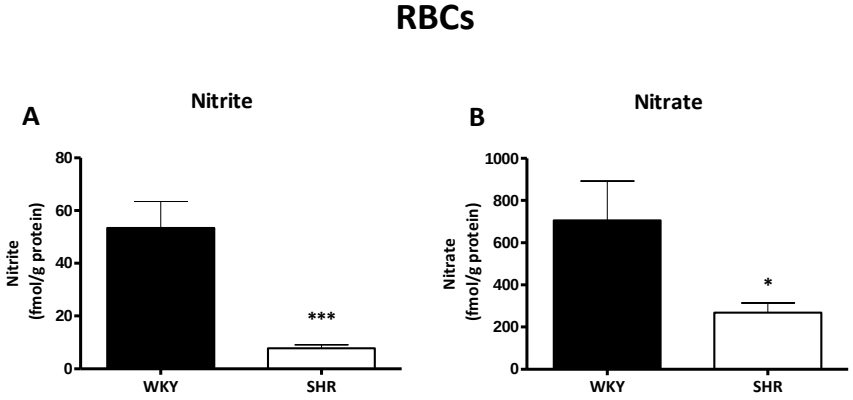


Figure 3.14: The levels of nitrite (A) and nitrate (B) in RBCs of WKY (n=4) and SHRs (n=7). Data shown as mean±SEM. Comparison between 2 groups using unpaired t-tests and shown as *** for $p<0.001$ and * for $p<0.05$.

3.6.2 Comparison of baseline NO_x levels in the tissues

Of all the organs the aorta had the highest levels of both nitrite and nitrate, followed by the heart and lung with 3x fewer levels in the kidney and liver. There were no differences in the levels of either of these anions between the WKY and SHRs.

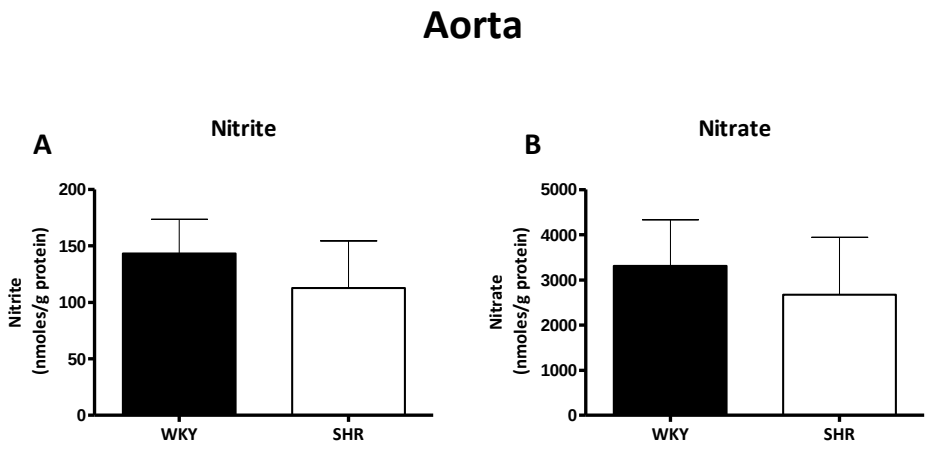


Figure 3.15: The levels of nitrite (A) and nitrate (B) in the aortas of WKY (n=15) and SHRs (n=12). Data shown as mean±SEM. Comparison between 2 groups made using unpaired t-tests.

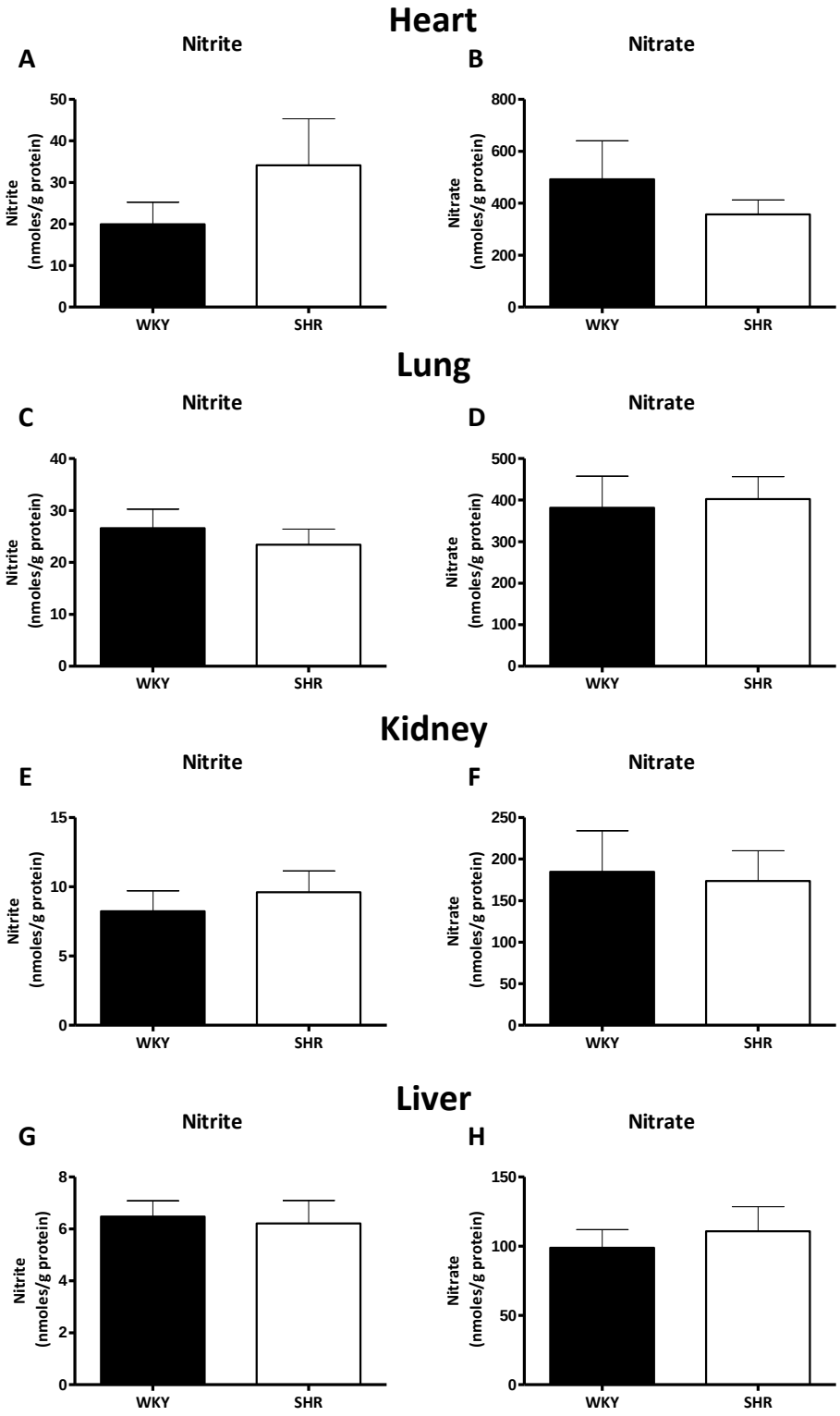


Figure 3.16: The levels of nitrite and nitrate in the heart, lung, kidney and liver (WKY n=10, SHR n=10). Data shown as mean±SEM. Comparison between 2 groups made using unpaired t-tests.

3.7 Summary

Feeding C57BL/6J wild type mice with the dietary anions had no effect on weight gain over time. Interestingly, whilst there were no statistical differences in food and water consumption both the highest dose of nitrite and nitrate did appear to be associated with increases in food and water consumption.

In healthy WT animals inorganic nitrite caused dose-dependent elevations in plasma nitrite, but no changes in plasma nitrate concentration. Administration of dietary inorganic nitrate caused dose-dependent elevations in both plasma nitrite and plasma nitrate. The fold elevations in plasma nitrite (seen with the 15 and 45mM nitrate doses) are similar to the fold elevations reported in healthy volunteer studies in which functional changes in blood pressure are seen.

To investigate the effect that CVD has on the distribution of these 2 anions the ApoE KO model was used. As with the WT mice the mice were administered the drug in the drinking water for 2 weeks. The primary observation made was that basal levels of nitrate were significantly reduced in the ApoE KO compared to the WT across most of the tissues. The specific tissues affected were the plasma, RBCs, kidney, heart, liver aorta and lungs. For each of these tissues the most effective way in restoring nitrate and nitrite levels to baseline was through the administration of a dietary inorganic nitrate (15mM) intervention. This intervention consistently elevated nitrate levels above baseline in all the tissues.

The nitrite profiles across the tissues are similar. Basal levels of nitrite appear not to be affected by early stage atherosclerosis. In addition, administration of inorganic nitrite does not significantly elevate nitrite across the tissues in either the WT or the ApoE KO mice. There are, however, 2 notable exceptions. In the RBCs, nitrite levels are significantly elevated in both genotypes. Interestingly in the mesentery, nitrite is significantly elevated in the ApoE KO compared to the WT animals. These results suggest perhaps some selective uptake of nitrite in these tissues.

Furthermore hypertension appeared to have no effect on the baseline distribution of nitrite and nitrate across the organs examined. The RBCs did, however, show significantly reduced levels of both anions with hypertension. This is possibly due to enhanced nitrite reductase activity seen at the level of the RBC, which is discussed in detail in the next chapter.

Chapter 4:

Investigation of the nitrite reductase activity in models of cardiovascular disease and patients

4.1 Introduction

CVD is associated with a reduction in bioavailable NO. This reduction in part is due to dysfunction of the classical L-arginine/NOS mediated pathway of NO generation (Moncada *et al.*, 1991). Given the importance of NO in sustaining a healthy cardiovascular system much work currently focuses on identifying alternative strategies of NO generation. A recent point of focus has been assessing the possibility of utilizing nitrite and nitrate as endogenous sources of NO. NOS-independent formation of NO within the cardiovascular system was first reported in the ischaemic heart where it was demonstrated that endogenous nitrite was reduced to NO (Zweier *et al.*, 1995). At that time the authors speculated that the conditions generated were a consequence of hypoxia i.e. acidosis, which resulted in the reduction of nitrite to NO.

However, since that time it has been discovered that nitrite reductases operate to facilitate this reaction. In particular previous studies have suggested that XOR is an important nitrite reductase in the heart and kidney. Using ozone chemiluminescence it was reported that both rat and human myocardium under ischaemic conditions produce NO from nitrite that is dependent on XOR (Webb *et al.*, 2004a). Similarly in rat kidney the XOR inhibitor allopurinol profoundly attenuated nitrite reductase activity (Tripatara *et al.*, 2007).

In the previous investigations the pre-clinical studies involved subjecting organs (in vitro or in vivo) to acute insults that mimic CVD i.e. animals were healthy prior to acute insult. Whether nitrite reductase activity is altered by disease is unknown. This is an important point to investigate since the utility of nitrite as a therapeutic in CVD might offer potential not only in acute scenarios such as I/R injury where nitrite reductase activity is upregulated but also in progressive CVD where environmental conditions are normal, such as hypertension and atherosclerosis. Both of these diseases are of particular interest with respect to the therapeutic potential of nitrite since both diseases have been associated with increased expression and activity of XOR (White *et al.*, 1996b; Laakso *et al.*, 1998b).

Therefore in this set of experiments the nitrite reductase activity in models of hypertension (SHR) and atherosclerosis (ApoE KO) have been investigated and compared with the nitrite reductase activity of the respective healthy controls i.e. WKY rats and C57BL/6J wild type mice respectively. In addition key sites/tissue for nitrite reductase activity have been assessed that are pertinent for XOR i.e. the liver, vascular tissue and the erythrocyte, which are all sites of prominent nitrite reductase capacity (Webb *et al.*, 2004a; Duranski *et al.*, 2005; Webb *et al.*, 2008a).

4.2 Nitrite reductase activity in atherosclerosis

4.2.1 Nitrite reductase activity in the liver

The nitrite reductase activity of liver homogenates was significantly enhanced across the full concentration range of nitrite tested in ApoE KO vs. WT mice at pH 7.4 (figure 4.1A). A similar trend was also evident at pH 6.8 (figure 4.1B).

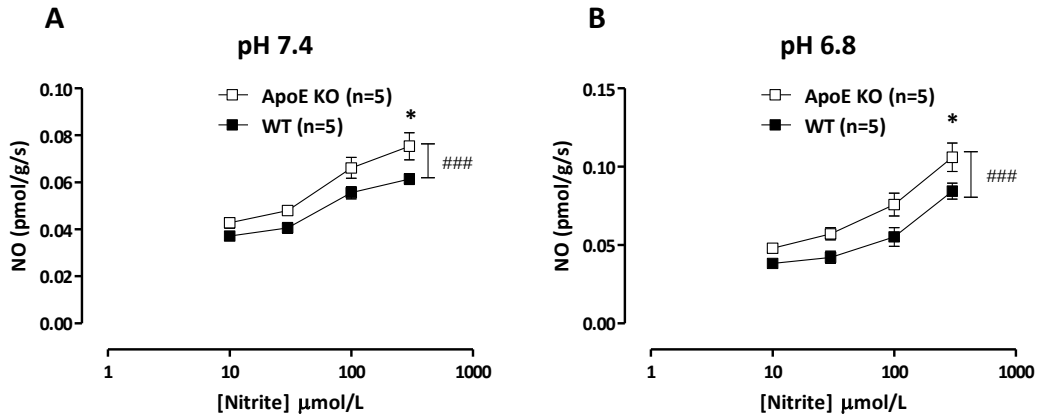


Figure 4.1: Nitrite reductase activity of mouse liver homogenates. Comparison of the nitrite reductase activity at pH 7.4 ApoE KO vs. WT (A) and pH 6.8 ApoE KO vs. WT (B). Statistical significance between concentration response curves determined using 2-way ANOVA and shown as ### for $p<0.001$ followed by Bonferroni post tests shown as * for $p<0.05$.

4.2.2 Mechanism of nitrite reductase activity in the liver

Incubation of liver homogenates with allopurinol did not alter the extent of nitrite-derived NO generation from liver homogenates of WT mice at pH 7.4 (figure 4.2A) or pH 6.8 (figure 4.2B). In contrast, allopurinol significantly inhibited nitrite reduction at pH 7.4 (figure 4.2C) and pH 6.8 (figure 4.2D) in liver homogenates from ApoE KO mice.

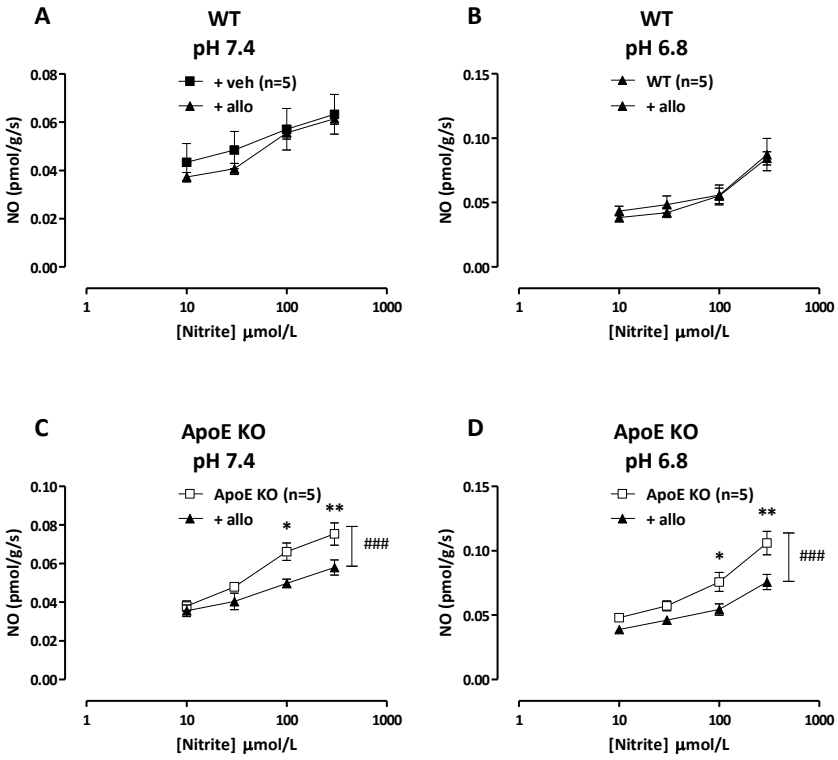


Figure 4.2: XOR-dependent nitrite reductase activity of mouse liver homogenates. Both liver types were tested in the absence and presence of the XOR inhibitor allopurinol (100µmol/L) for WT (A + B) and ApoE KO (C + D) mice. Data are expressed as mean±SEM with n indicated on the graphs. Statistical significance between concentration response curves determined using repeated measures 2-way ANOVA and shown as ### for $p<0.001$ followed by Bonferroni post tests shown as * for $p<0.05$ and ** for $p<0.01$.

4.2.3 Nitrite reductase activity in the RBCs

The nitrite reductase activity of mouse RBCs was significantly enhanced across the full concentration range of nitrite tested in ApoE KO vs. WT mice at pH 7.4 (figure 4.3A). A similar trend was also evident at pH 6.8 (figure 4.3B).

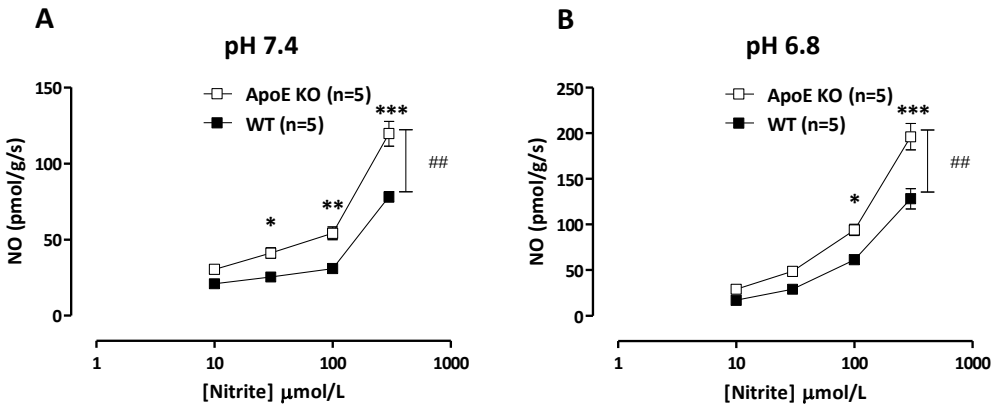


Figure 4.3: Nitrite reductase activity of mouse RBCs. Comparison of the nitrite reductase activity at pH 7.4 ApoE KO vs. WT (A) and pH 6.8 ApoE KO vs. WT (B). Statistical significance between concentration response curves determined using 2-way ANOVA and shown as ## for $p<0.01$ followed by Bonferroni post tests shown as *** for $p<0.001$, ** for $p<0.01$ and * for $p<0.05$.

4.2.4 Mechanism of nitrite reductase activity in the RBCs

Incubation of mouse RBCs with allopurinol did not alter the extent of nitrite-derived NO generation from RBCs of WT mice at pH 7.4 (figure 4.4A) or pH 6.8 (figure 4.4B). In contrast, allopurinol significantly inhibited nitrite reduction at pH 7.4 (figure 4.4C) and pH 6.8 (figure 4.4D) in RBCs from ApoE KO mice.

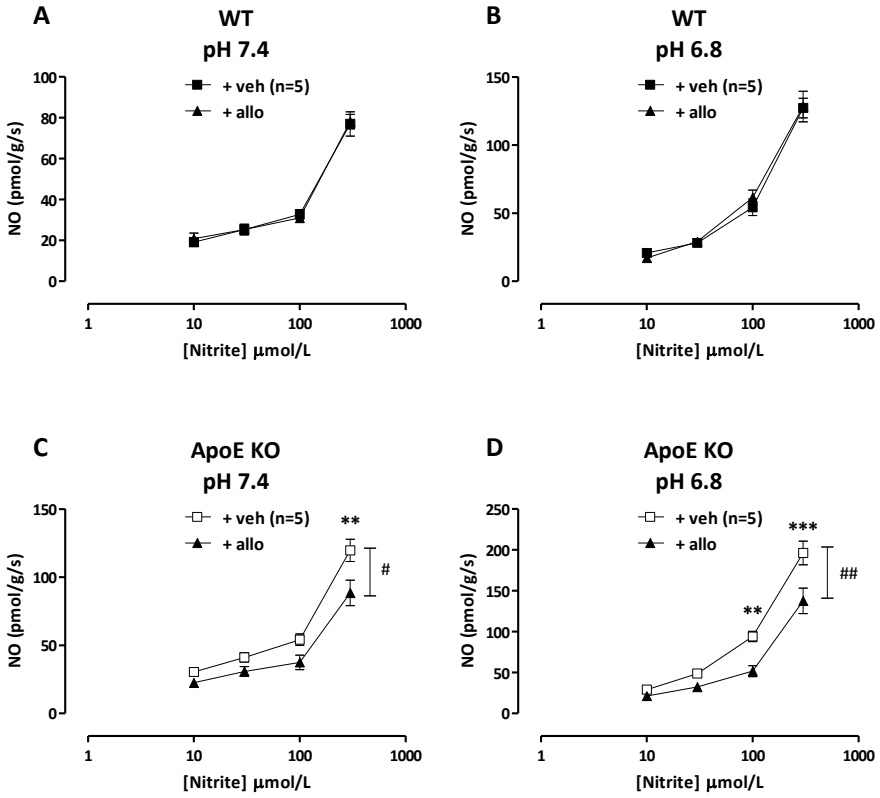


Figure 4.4: XOR-dependent nitrite reductase activity of mouse RBCs. Both RBC types were tested in the absence and presence of the XOR inhibitor allopurinol (100µmol/L) for WT (A + B) and ApoE KO (C + D) mice. Data are expressed as mean±SEM with n indicated on the graphs. Statistical significance between concentration response curves determined using 2-way ANOVA and shown as ### for p<0.001 and # for p<0.05 followed by Bonferroni post tests shown as *** for p<0.001 and ** for p<0.01.

4.3 Expression of XOR in atherosclerosis

4.3.1 Expression of XOR in the liver

XOR expression in mouse liver homogenates from ApoE KO mice was significantly enhanced compared to WT mice (figure 4.5A + B).

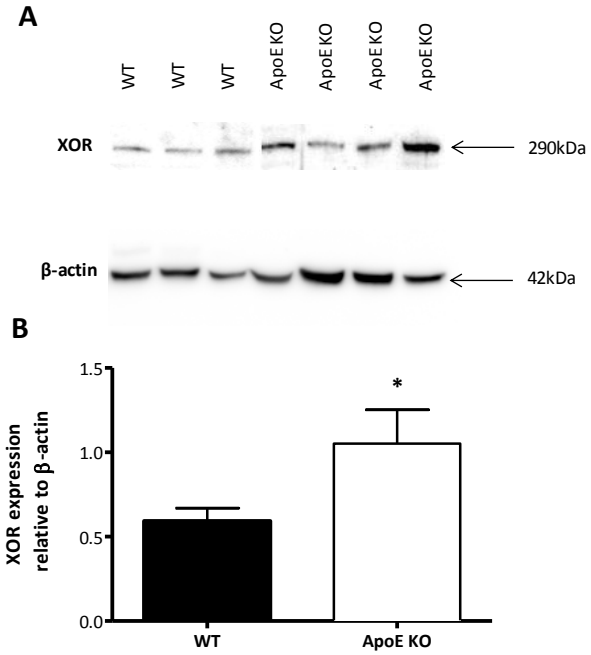


Figure 4.5: XOR expression levels of mouse liver homogenates for WT (n=8) and ApoE KO (n=8) mice as assessed by western blotting. Expression quantified by densitometry (normalized to control). Data shown as mean±SEM. Comparison between 2 groups using unpaired t-tests and shown as *p<0.05.

4.3.2 Expression of XOR in the RBCs

XOR expression in mouse RBCs from ApoE KO mice was significantly enhanced compared to WT mice (figure 4.6A + B).

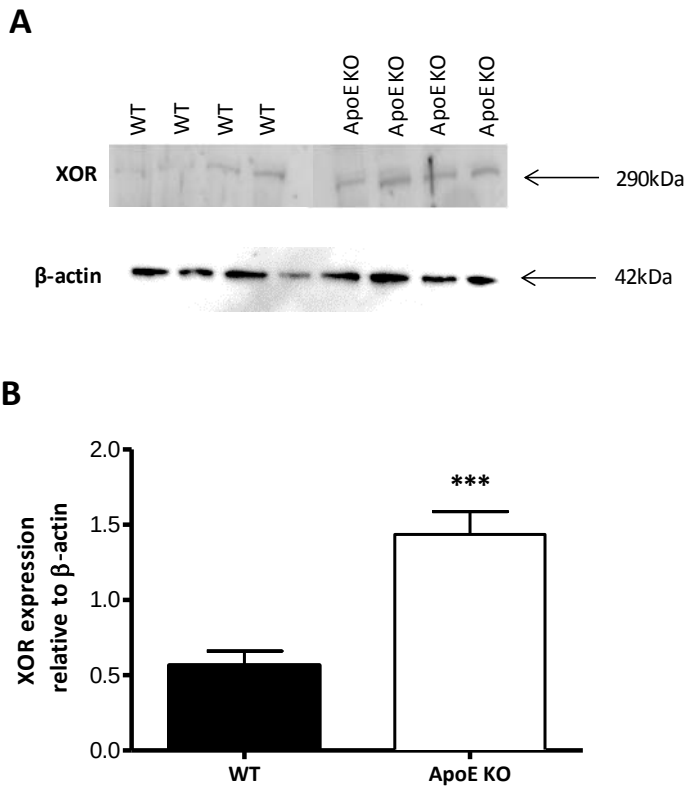


Figure 4.6: XOR expression levels of mouse RBCs for WT (n=8) and ApoE KO (n=8) mice as assessed by western blotting. Expression quantified by densitometry (normalized to control). Data shown as mean±SEM. Comparison between 2 groups using unpaired t-tests and shown as ***p<0.001.

4.4 Nitrite reductase activity in pre-clinical models of hypertension

4.4.1 Nitrite reductase activity in aorta

Nitrite-derived NO formation was similar in aortic homogenates of WKY (figure 4.7A) and SHR (figure 4.7B). Furthermore XOR inhibition had no effect on the ability of the blood vessel wall to reduce nitrite to NO in either the WKY (figure 4.7A) or the SHR (figure 4.7B).

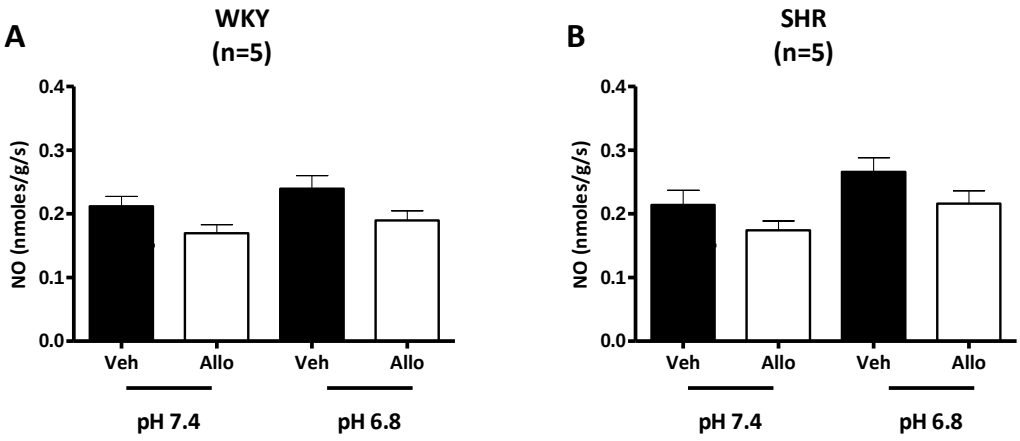


Figure 4.7: Nitrite reductase activity of aortic homogenates of WKY at pH 7.4 and 6.8 (A) and SHR at pH 7.4 and 6.8 (B) in the presence of nitrite (300µmol/L) and allopurinol (100µmol/L). Data shown as mean±SEM with n indicated on graphs. No significant differences in nitrite reductase activity using 1-way ANOVA.

4.4.2 Nitrite reductase activity in RBCs

Nitrite reductase activity of purified RBCs was significantly enhanced in SHR vs. WKY at pH 7.4 (Figure 4.8A). A similar trend was seen at pH 6.8 but this did not reach statistical significance (Figure 4.8B).

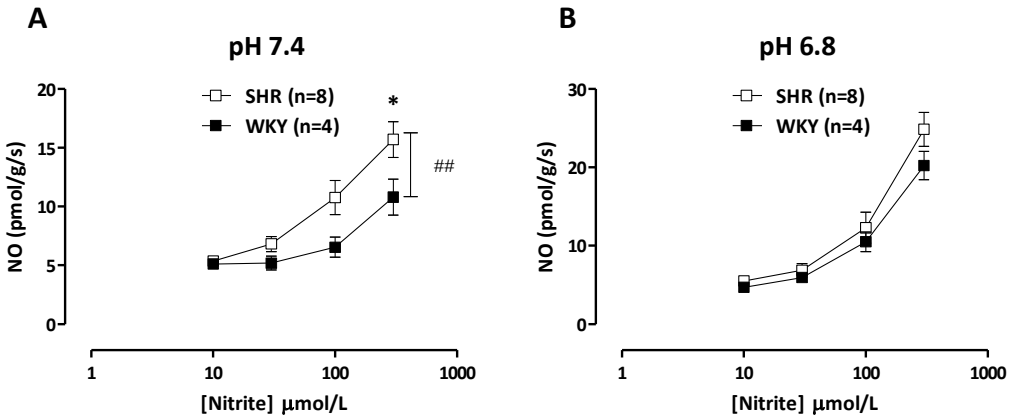


Figure 4.8: Nitrite reductase activity of rat RBCs. Comparison of the nitrite reductase activity at pH 7.4 WKY vs. SHR (A) and pH 6.8 WKY vs. SHR (B). Data shown as mean±SEM. Statistical significance between concentration response curves determined using 2-way ANOVA and shown as ## for $p<0.01$ followed by Bonferroni post tests shown as * for $p<0.05$.

4.4.3 Mechanism of nitrite reductase activity in RBCs

Incubation of RBCs with allopurinol did not alter the extent of nitrite-derived NO generation of RBCs of WKY animals at pH 7.4 (figure 4.9A) or pH 6.8 (figure 4.9B). In contrast, allopurinol significantly inhibited nitrite reduction at pH 7.4 (Figure 4.9C) and pH 6.8 (Figure 4.9D) in RBCs of SHRs.

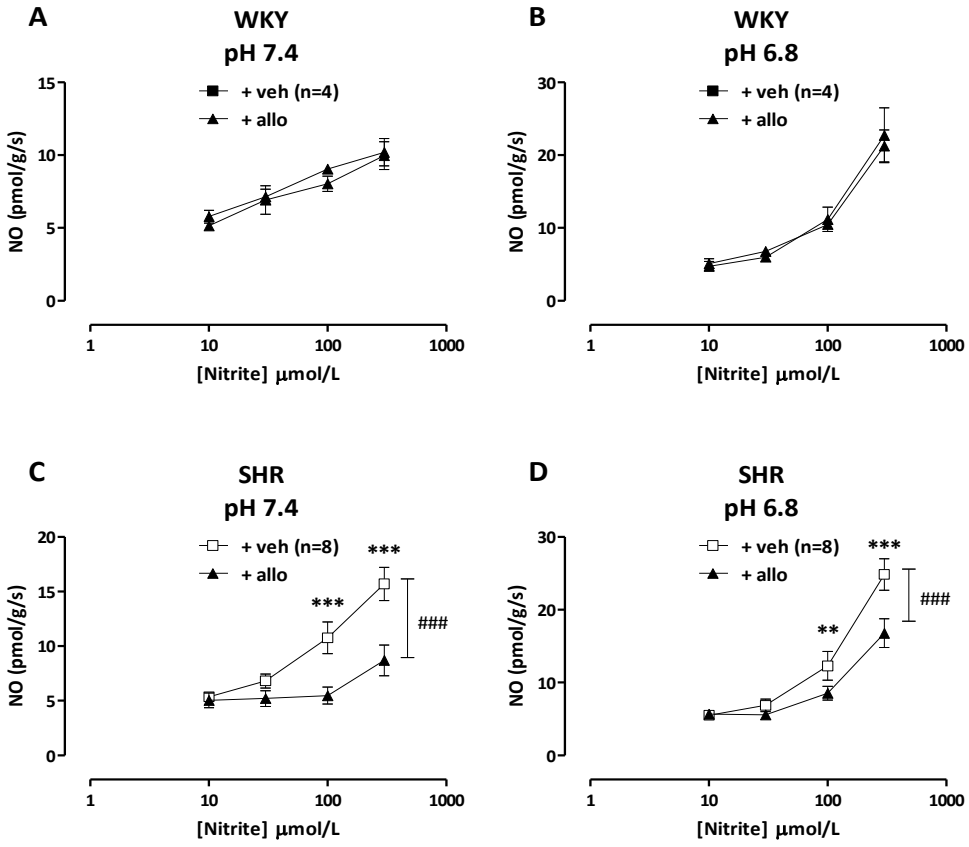


Figure 4.9: XOR-dependent nitrite reductase activity of rat RBCs. Both RBC types were tested in the absence and presence of the XOR inhibitor allopurinol (allo, 100 µmol/L) for WKY (A + B) and SHR (C + D). Data are expressed as mean±SEM with n indicated on the graphs. Statistical significance between concentration response curves determined using 2-way ANOVA and shown as ### for $p<0.001$ followed by Bonferroni post tests shown as ** for $p<0.01$ and *** for $p<0.001$.

4.5 Expression of XOR in WKY vs. SHRs

There was significantly enhanced XOR expression in rat liver homogenates from SHRs compared to WKY rats (figure 4.10).

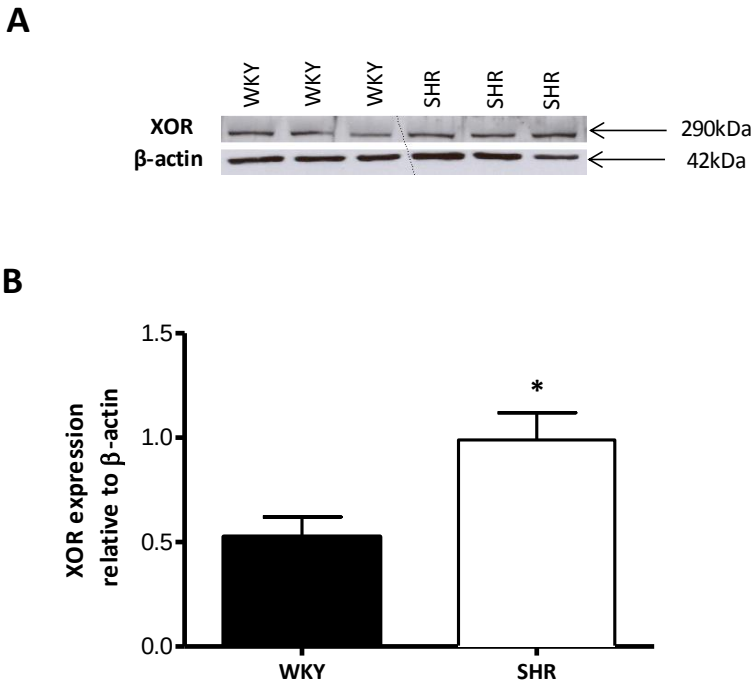


Figure 4.10: XOR expression levels of liver homogenates. Comparison between WKY (n=7) and SHR (n=8). Data shown as mean±SEM. Comparison between 2 groups assessed using unpaired t-tests.

There was no difference in XOR expression in rat aorta homogenates from SHRs compared to WKY rats (figure 4.11).

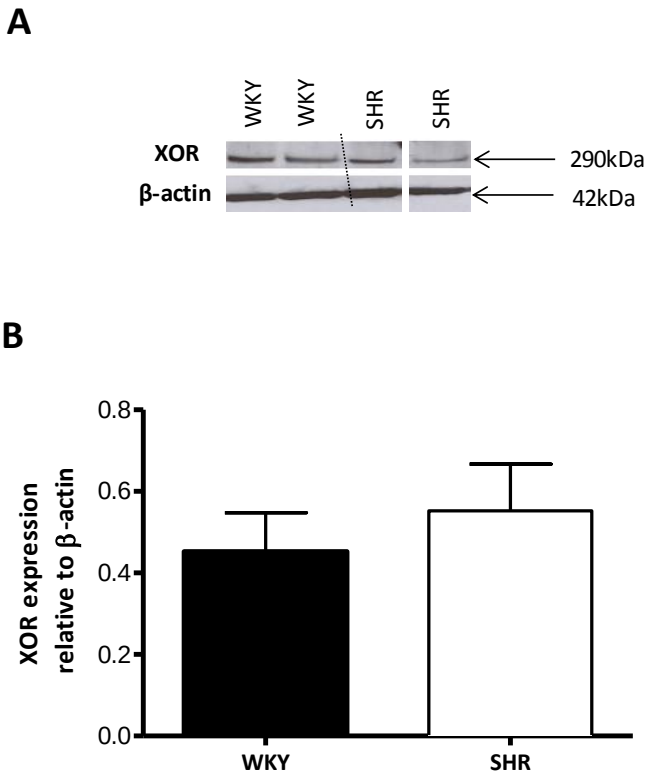


Figure 4.11: XOR expression levels of mouse aorta homogenates. Comparison between WKY (n=8) and SHR (n=6). Data shown as mean±SEM. Comparison between 2 groups assessed using unpaired t-tests.

4.5.3 RBCs

An approximate doubling of erythrocytic XOR expression was observed in the RBCs isolated from SHRs compared to control WKY animals (figure 4.12).

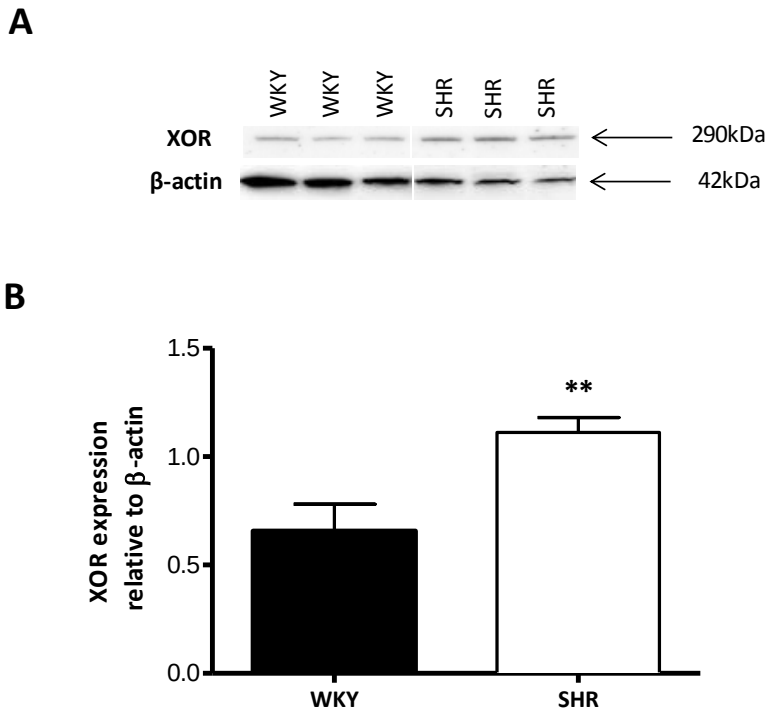


Figure 4.12: Expression of erythrocytic XOR determined by western blotting. Comparison between WKY (n=4) and SHR (n=6). Data are expressed as mean±SEM. Comparison between 2 groups using unpaired t tests and shown as **p<0.01.

4.6 Nitrite reductase activity in human hypertension

In a proof-of-principle study, grade 1 drug-naïve hypertensive subjects (determined from previous 24-hour ambulatory blood pressure measurements conducted <1 month before study date) were randomized in an open-label crossover design to receive 250ml of inorganic nitrate-rich beetroot juice (dietary nitrate; James White Drinks, UK or an equal volume of water (placebo; Zepbrook Ltd, UK)). On each visit clinic blood pressure measurements were made for 1 hour to provide baseline blood pressure before intervention. The baseline hemodynamic/biochemical parameters of the volunteers at screening are shown in table 4.2.

Parameter	Normotensives	Hypertensives
Subjects, n	20	15
Systolic ABP, mmHg	110.1±3.4	151.5±1.5
Diastolic ABP, mmHg	70.1±2.3	89.7±2.2

Table 4.1: Hemodynamic/biochemical parameters of volunteers at screening. Data are shown as mean±SEM values. ABP measurements were taken with a Spacelabs 90207 (Spacelabs Healthcare, Issaquah, WA). Ambulatory blood pressure (ABP) values adjusted as per British Hypertension Society guidelines (Williams *et al.*, 2004).

4.6.1 Nitrite reductase activity in RBCs

Nitrite reductase activity of purified RBCs was significantly enhanced in hypertensive vs. normotensive patients at pH 7.4 (figure 4.13A) and pH 6.8 (figure 4.13B).

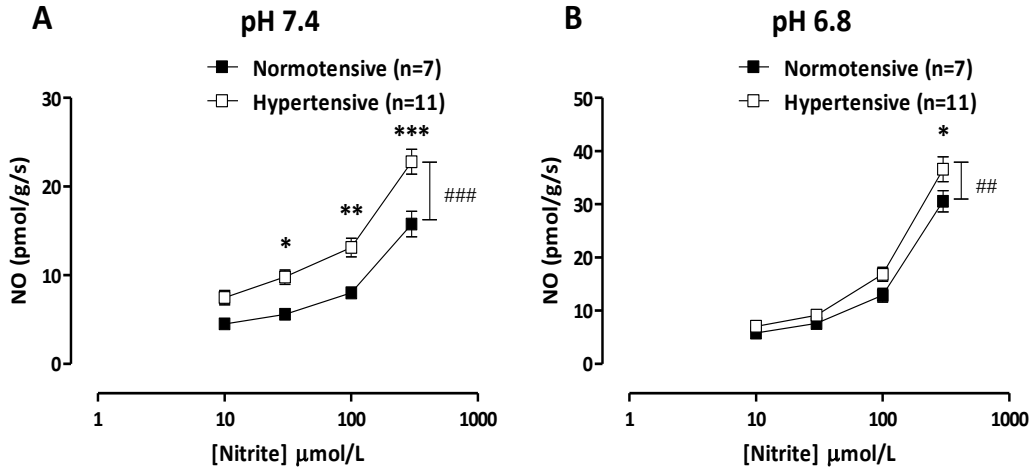


Figure 4.13: Nitrite reductase activity of patient RBCs. Comparison of the nitrite reductase activity at pH 7.4 normotensive vs. hypertensive (A) and pH 6.8 normotensive vs. hypertensive (B). Data shown as mean±SEM. Statistical significance between concentration response curves determined using 2-way ANOVA and shown as ### for p<0.001 and ## for p<0.01 followed by Bonferroni post tests shown as *** for p<0.001, ** for p<0.01 and * for p<0.05.

4.6.2 Mechanism of nitrite reductase activity in RBCs

Previous studies with RBCs of human normotensives show no effect of allopurinol on nitrite reductase activity (Webb *et al.*, 2008a). However, incubation of RBCs with allopurinol significantly inhibited nitrite-derived NO generation of RBCs from hypertensive patients at pH 7.4 (figure 4.14A) and 6.8 (figure 4.14B).

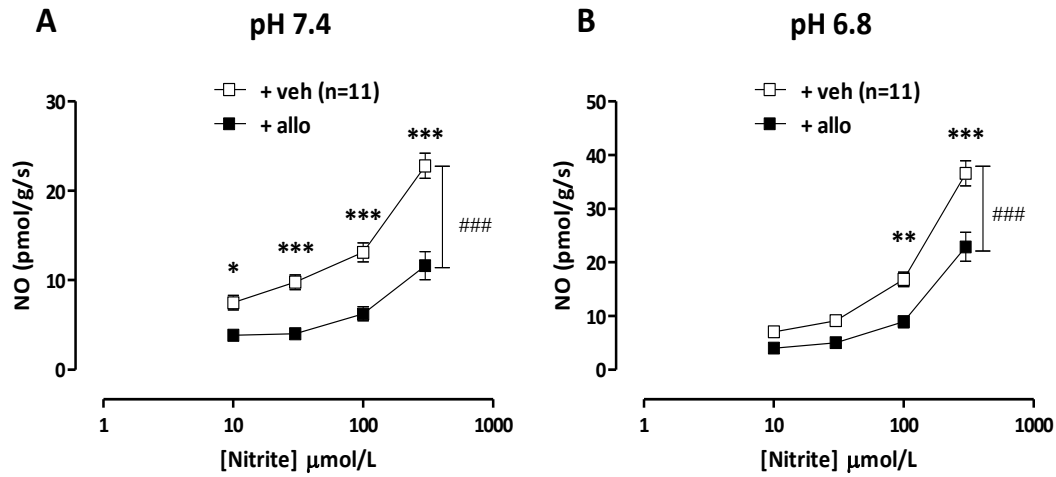


Figure 4.14: XOR facilitates nitrite-derived NO generation by erythrocytes of hypertensives. Comparison of the nitrite reductase activity of purified erythrocytes at pH 7.4 (A) and 6.8 (B) in the presence of vehicle or allopurinol (100µmol/L). Data are expressed as mean±SEM with n indicated on the graphs. Statistical significance between concentration response curves determined using 2-way ANOVA and shown as ### for $p<0.001$ followed by Bonferroni post tests shown as *** for $p<0.001$, ** for $p<0.01$ and * for $p<0.05$.

4.7 Expression of XOR in human hypertension

4.7.1 RBCs

Raised nitrite reductase activity seen in patient erythrocytes was associated with significantly higher levels of XOR expression in erythrocytes of hypertensive patients compared to normotensive volunteers (figure 4.15).

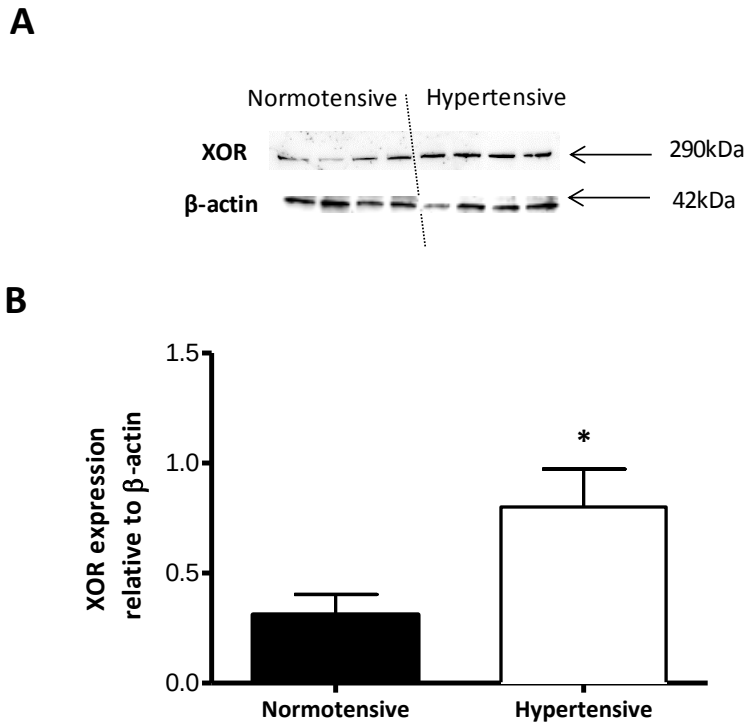


Figure 4.15: The expression of XOR of erythrocytes purified from the blood of normotensive and hypertensive volunteers (n=4 for each) determined by western blotting. Data expressed as mean±SEM. Comparison between 2 groups using unpaired t tests and shown as *p<0.05.

4.8 Summary

Within this chapter the potential significance of XOR in CVD has been investigated. Firstly using a mouse model of atherosclerosis it was shown that liver homogenates and RBCs taken from ApoE KO mice have enhanced nitrite reductase activity compared to WT controls. Furthermore, this enhanced activity was significantly attenuated by the XOR inhibitor allopurinol in both tissues. Western blotting demonstrated that XOR expression was raised in both the liver and RBCs of ApoE KO compared to WT mice.

The second model of CVD used was the spontaneously hypertensive rat (SHR). Initially nitrite reduction was examined at the level of the blood vessel wall since in the literature there is evidence to suggest that key nitrite reductase activity occurs in this compartment (Li *et al.*, 2008). While nitrite reductase activity of aortic homogenates was evidenced no difference in activity between the SHR and the WKY control was found. Moreover, this activity was not affected by XOR inhibition. In contrast washed RBCs showed significantly enhanced nitrite reduction in the SHR compared to the WKY. Furthermore XOR inhibition attenuated nitrite-derived NO generation in the RBCs of SHRs but had no impact in the WKY rats. Western blotting of tissues suggested that this increased activity was likely due to increased XOR expression.

Erythrocytes isolated from the blood of 11 grade 1 hypertensive volunteers were assessed for nitrite reductase activity and compared to normotensive controls. Nitrite-derived NO generation was significantly elevated in the hypertensive erythrocytes compared to the normotensive controls. Moreover, as seen with the rat RBCs, this increased activity was complemented by higher XOR expression in the hypertensive erythrocytes. Finally the nitrite reductase activity in the hypertensive erythrocytes was significantly inhibited by allopurinol. These findings fit well with previous work from the lab, which showed that at pH 7.4 allopurinol did not have an effect on nitrite-derived NO generation by RBCs collected from healthy volunteers (Webb *et al.*, 2008a).

Chapter 5:

Investigation of the effect of dietary nitrate on inflammatory cell recruitment

5.1 Introduction

Acute inflammation is characterized by migration of inflammatory cells to the site of injury. NO which is constantly being produced by the endothelium, exerts important anti-inflammatory effects on this response mainly through prevention of leukocyte activation and adhesion (Kubes *et al.*, 1991; Gaboury *et al.*, 1993). Recent research has uncovered the potential for using inorganic nitrite and nitrate (either from endogenous or dietary sources) as alternative sources of NO (Lundberg *et al.*, 2008). At the time of starting this work, whether dietary nitrate might influence inflammatory cell recruitment was unknown. To address this issue the effect of a dietary nitrate regimen on inflammatory cell recruitment induced by a range of stimuli (IL-1β, TNFα and zymosan) was investigated.

Zymosan promotes a well characterized inflammatory response that is due to triggering of the complement cascade (Rawal *et al.*, 1998; Mizuno *et al.*, 2009) resulting in a substantial neutrophil recruitment. Classically the response to zymosan mimics an acute hypersensitivity response, although activation of the complement cascade has been demonstrated in certain CVD states including ischaemic heart disease (Langlois *et al.*, 1988; Yasuda *et al.*, 1990; Pedersen *et al.*, 2004).

First identified in 1975 (Carswell *et al.*, 1975) TNFα is a potent cytokine that promotes inflammation and tissue destruction in rheumatic/immune mediated diseases. TNFα effects paracrine or autocrine regulation of leukocytes and endothelial cells and therefore serves as an important regulator of the inflammatory response. TNFα is important in CVD as it has been shown to increase in the myocardium after experimental ischaemia and reperfusion (Gulick *et al.*, 1989; Herskowitz *et al.*, 1995) and clinical myocardial infarction (Latini *et al.*, 1994).

IL-1β was first described in 1972 as a lymphocyte-activating factor (Gery *et al.*, 1972) and is a trigger for neutrophil and monocyte recruitment. It has been shown to be involved in myocardial I/R injury (Li *et al.*, 2004b) and ischaemic heart disease particularly coronary artery disease (Hasdai *et al.*, 1996).

5.2 The effect of inflammatory stimuli on cell recruitment

In comparison to PBS, IL-1β and TNFα did not cause a substantial increase in the total number of cells recruited to the peritoneal cavity of WT mice over a 4h period (figure 5.1A). In contrast zymosan caused a substantial increase (~5-fold) in total cell count (figure 5.1A). After 24h these differences were still apparent although there was some evidence of the resolution of inflammation as the total cell number for zymosan had decreased to approximately $20x10^6$ from $30x10^6$ (figure 5.1B).

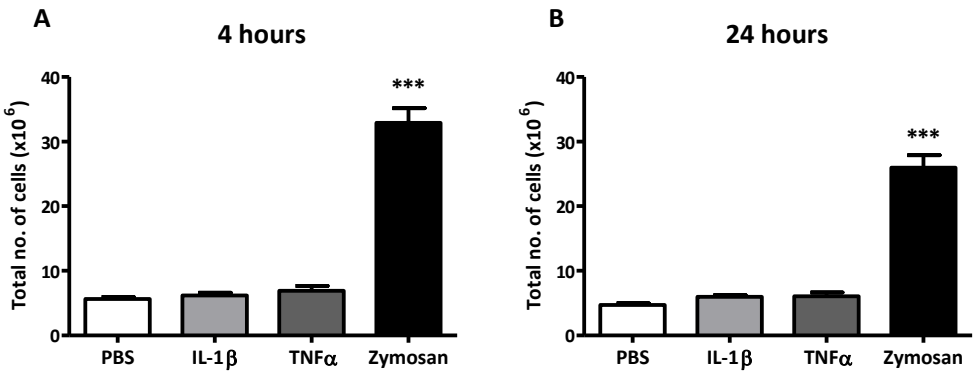


Figure 5.1: The effect of stimulus on total leukocyte count with PBS (n=9-10), IL-1β (n=7-10), TNFα (n=10) and zymosan (n=15-17) injected mice. Data shown as mean±SEM. A significant difference is shown as ***p<0.001 vs. PBS using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

5.3 The effect of inflammatory stimuli on type of leukocyte recruited

I.p. injection of control mice with PBS resulted in almost 100% of cells collected from the cavity being resident monocytes (figure 5.2A) with a small percentage being neutrophils (figure 5.2B) and inflammatory monocytes (figure 5.2C) 4h after the injection of stimulus. IL-1β and TNFα caused more of a response as a greater percentage of neutrophils (figure 5.2B) and inflammatory monocytes (figure 5.2C) was evident after 4h. The response to both IL-1β and TNFα was resolved after 24h as the profile for both stimuli reverted to the profile for PBS (figure 5.2E, F + G). In contrast, zymosan caused a substantial influx of neutrophils into the peritoneal cavity (figure 5.2C) resulting in very few resident ($0.42x10^6$; figure 5.2A + table 5.1) and inflammatory monocytes ($1.0x10^6$; figure 5.2C + table 5.1) being present. After 24h, inflammation was not completely resolved as neutrophil percentage was still high (figure 5.2F), which was also reflected in the high cell count (table 5.1), although resident monocyte (figure 5.2E) and inflammatory monocyte (figure 5.2G) percentages and cell counts were rising (table 5.1).

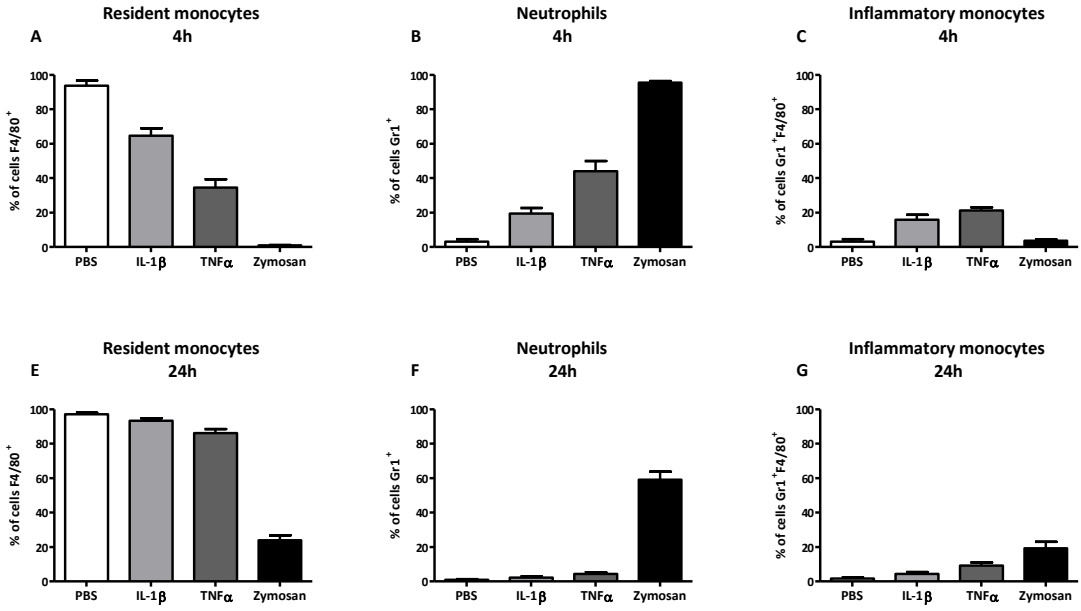


Figure 5.2: The effect of stimulus on cell type with PBS (n=9), IL-1β (n=7-8), TNFα (n=10) and zymosan (17) injected mice. Data shown as mean±SEM.

		Cell numbers (x10^6)			
Time point	**Stimulus**	**Total**	**Neutrophils**	**Resident Monocytes**	**Inflammatory Monocytes**
4h	PBS	5.95±0.33	0.20±0.12	5.53±0.23	0.22±0.12
4h	IL-1β	6.20±0.42	1.20±0.21	4.01±0.27	0.99±0.17
4h	TNFα	6.10±0.88	2.38±0.65	2.47±0.55	1.41±0.18
4h	Zymosan	32.91±2.26	30.23±1.36	0.42±0.12	1.03±0.31
24h	PBS	4.74±0.32	0.07±0.02	4.47±0.13	0.09±0.04
24h	IL-1β	5.97±0.26	0.13±0.04	5.58±0.09	0.24±0.06
24h	TNFα	6.25±0.71	0.23±0.05	4.87±0.50	0.47±0.11
24h	Zymosan	22.93±1.35	12.71±1.18	6.35±0.96	4.42±0.89

Table 5.1: Total leukocyte sub-type numbers in the peritoneal cavity.

5.4 The effect of inflammatory stimuli on MPO levels in the cell pellet

In comparison to PBS control MPO activity was raised for all stimuli 4h after the induction of inflammation (figure 5.3A). A substantial increase was seen with zymosan (figure 5.3A). After 24h activity was still raised in comparison to PBS although resolution of inflammation was evidenced with all stimuli as activity had decreased vs. 4h (figure 5.3B). The substantial difference between zymosan and PBS was still present after 24h (figure 5.3B).

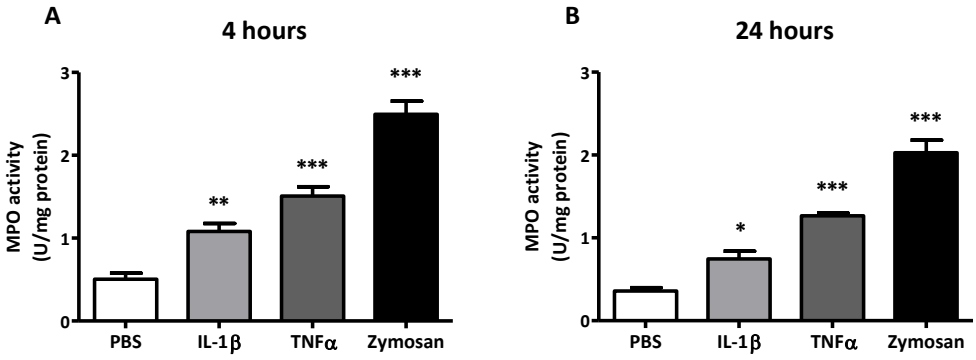


Figure 5.3: The effect of stimulus on MPO levels in the cell pellet with PBS, IL-1β, TNFα and zymosan (n=6) injected mice. Data shown as mean±SEM. A significant difference is shown as ***p<0.001, **p<0.01 or *p<0.05 vs. PBS using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

5.5 The effect of inflammatory stimuli on MPO levels in the mesentery

In comparison to PBS control MPO level was raised for all stimuli 4h after the induction of inflammation (figure 5.4A). A substantial increase was seen with zymosan (figure 5.4A). After 24h, activity was still raised in comparison to PBS although resolution of inflammation was evidenced with all stimuli as activity had decreased (figure 5.4B). The substantial difference between zymosan and PBS was still present after 24h (figure 5.4B).

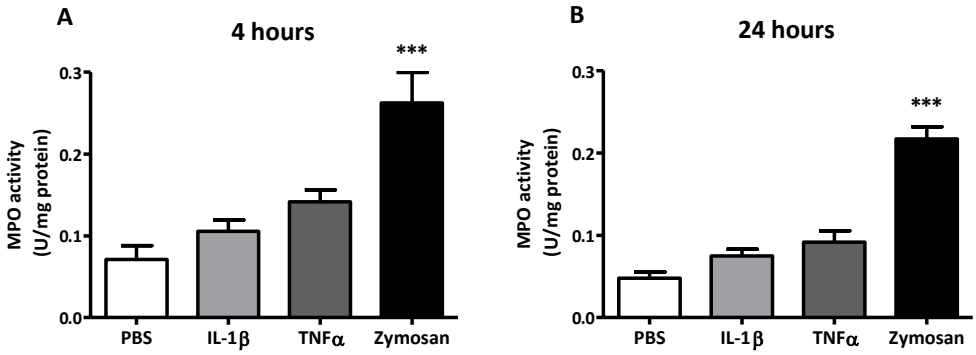


Figure 5.4: The effect of stimulus on MPO levels in the mesentery with PBS, IL-1β, TNFα and zymosan (n=6-10) injected mice. Data shown as mean±SEM. A significant difference is shown as ***p<0.001 vs. PBS using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

5.6 The effect of dietary nitrate on total cell number following i.p. injection of inflammatory stimuli

Since previous studies from chapter 3 demonstrated that only dietary nitrate supplementation was effective in elevating tissue levels of nitrite the effect of dietary nitrate supplementation on cell recruitment was investigated.

Dietary nitrate supplementation had no effect on total leukocyte count in the peritoneal lavage fluid collected from PBS control injected mice 4 (figure 5.5A) and 24h (figure 5.5B) following injection.

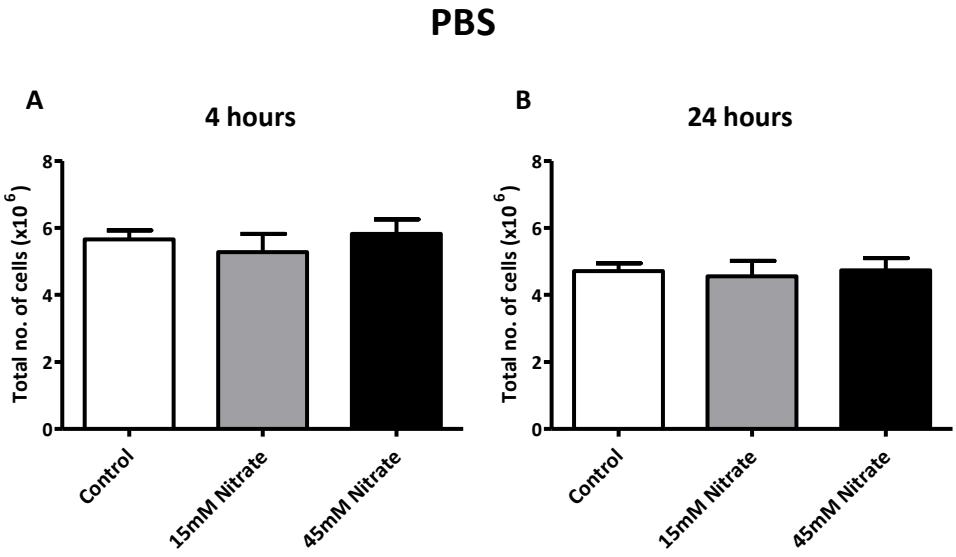


Figure 5.5: The effect of dietary nitrate on total leukocyte count 4 (A; n=9-10) and 24h (B; n=10) after i.p. injection of PBS (0.5ml). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Similarly there was no change in cell number with dietary nitrate treatment in IL-1β injected mice at 4 or 24h (figure 5.6A + B).

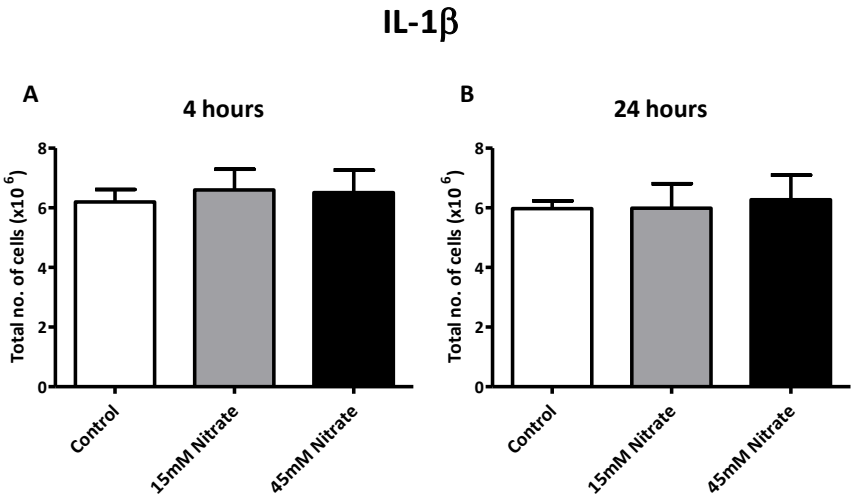


Figure 5.6: The effect of dietary nitrate on total leukocyte count 4 (A; n=8-10) and 24h (B; n=7) after i.p. injection of IL-1β (5ng). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

There did appear to be a trend to reduce total leukocyte count in the peritoneal lavage fluid collected from TNFα injected mice at 4 (figure 5.7A) and 24h (figure 5.7B) after the injection of stimulus. This reached significance at 4 but not 24h.

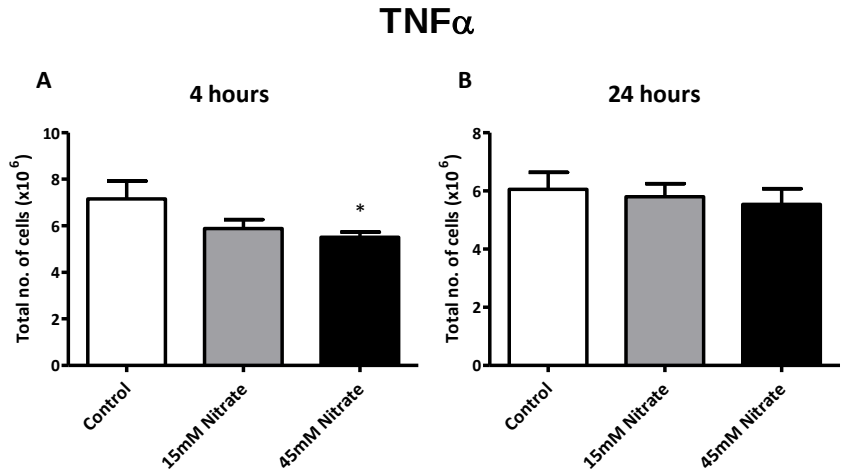


Figure 5.7: The effect of dietary nitrate on total leukocyte count 4 (A; n=9-10) and 24h (B; n=10) after i.p. injection of TNFα (300ng). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

In contrast significant reductions in total leukocyte count were evident in nitrate supplemented mice compared to control in response to zymosan at both 4 (figure 5.8A) and 24h (figure 5.8B), however this was only statistically significant at the higher 45mM concentration.

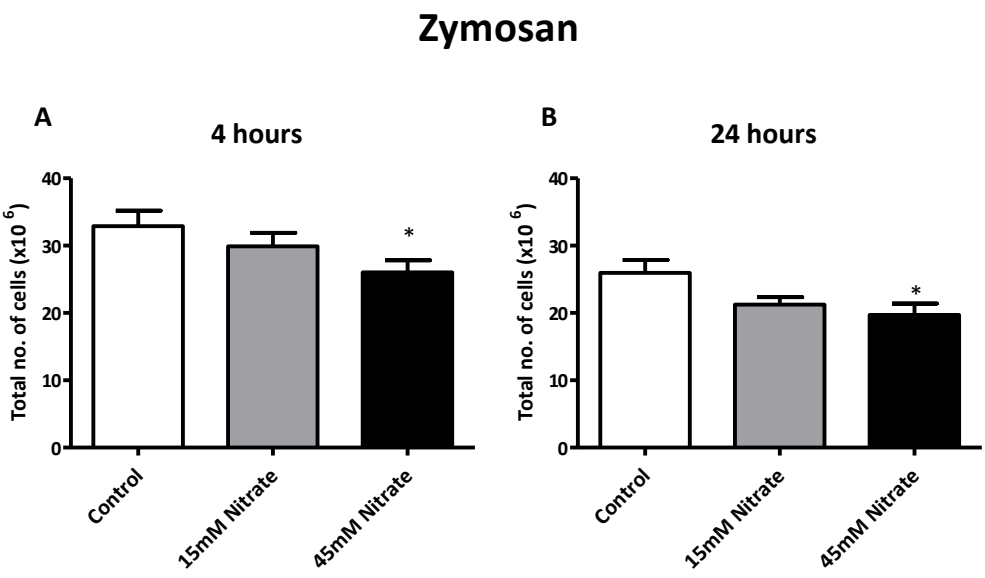


Figure 5.8: The effect of dietary nitrate on total leukocyte count 4 (A; n=15-17) and 24h (B; n=17) after i.p. injection of zymosan (1mg). Data shown as mean±SEM. A significant difference is shown as *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

5.7 The effect of dietary nitrate treatment on cell type following i.p. injection of inflammatory stimuli

Dietary nitrate treatment had no effect on inflammatory cell type in PBS control injected mice with respect to resident monocytes (figure 5.9A + D), neutrophils (figure 5.9B + E) and inflammatory monocytes (figure 5.9C + F) at 4 or 24h after the injection of PBS.

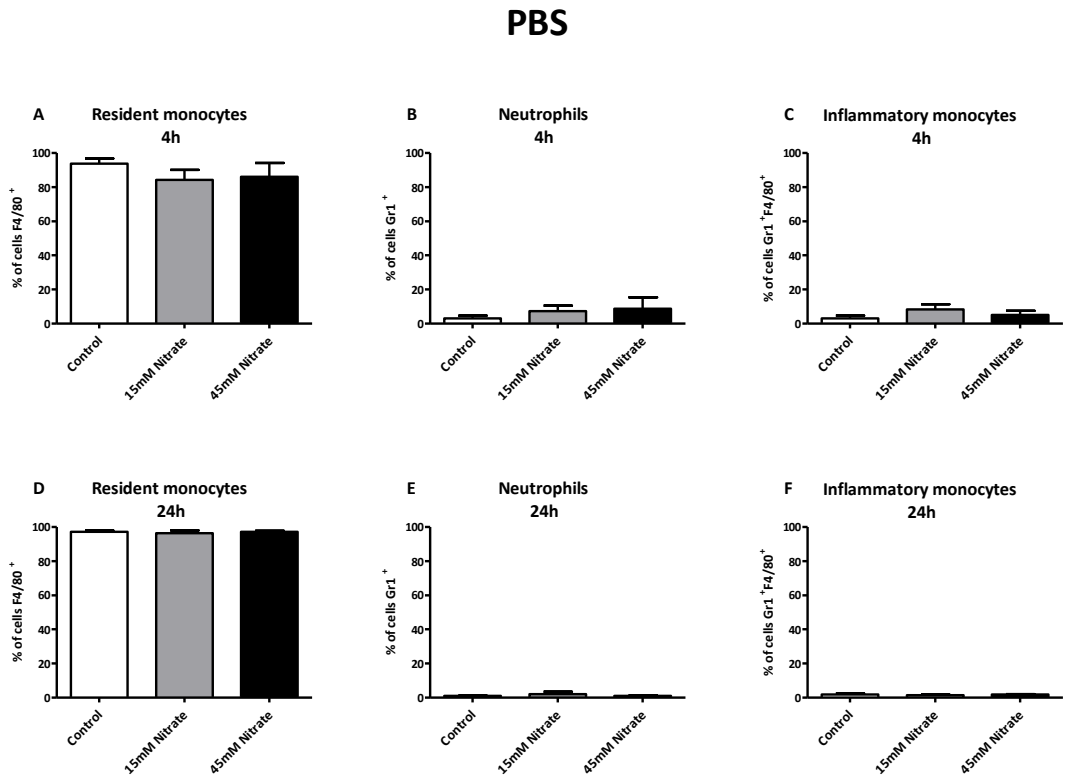


Figure 5.9: The effect of dietary nitrate on resident monocytes (A + D; n=9-10), neutrophils (B + E; n=9-10) and inflammatory monocytes (C + F; n=9-10) 4 and 24h after i.p. injection of PBS (0.5ml). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Dietary nitrate treatment had no effect on IL-1β-induced changes in resident monocytes (figure 5.10A + D), neutrophils (figure 5.10B + E) and inflammatory monocytes (figure 5.10C + F) either at 4 or 24h after the injection of IL-1β.

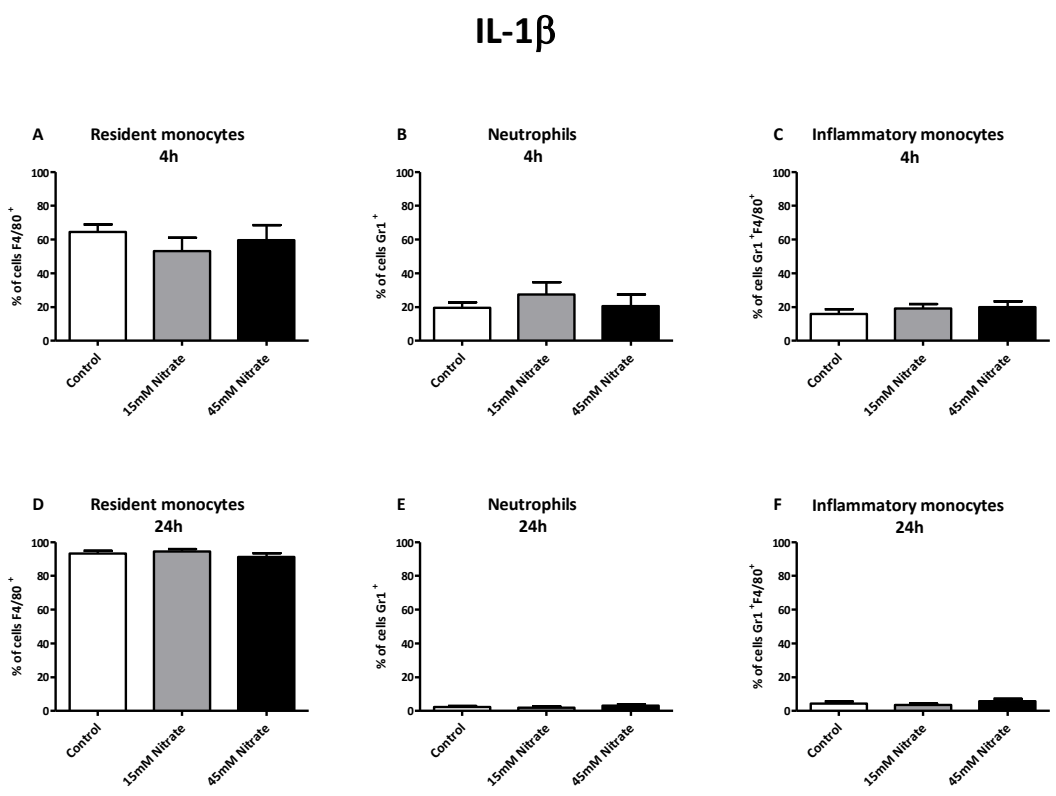


Figure 5.10: The effect of dietary nitrate on resident monocytes (A + D; n=7-8), neutrophils (B + E; n=7-8) and inflammatory monocytes (C + F; n=7-8) 4 and 24h after i.p. injection of IL-1β (5ng). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Dietary nitrate treatment also had no effect on TNFα-induced changes in resident monocytes (figure 5.11A + D), neutrophils (figure 5.11B + E) and inflammatory monocytes (figure 5.11C + F) at either 4 or 24h after the injection of TNFα.

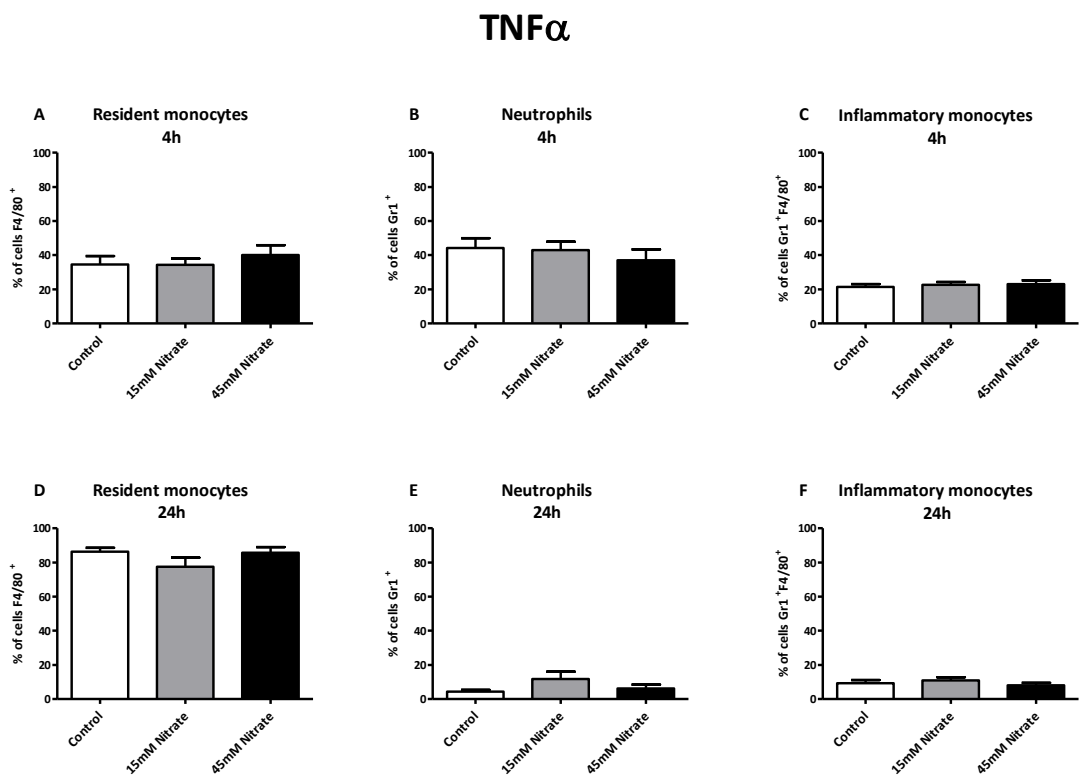


Figure 5.11: The effect of dietary nitrate on resident monocytes (A + D; n=9-10), neutrophils (B + E; n=9-10) and inflammatory monocytes (C + F; n=9-10) 4 and 24h after i.p. injection of TNFα (300ng). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Dietary nitrate treatment had no effect on zymosan-induced changes in resident monocytes (figure 5.12A + D), neutrophils (figure 5.12B + E) and inflammatory monocytes (figure 5.12C + F) at either 4 or 24h after the injection of zymosan.

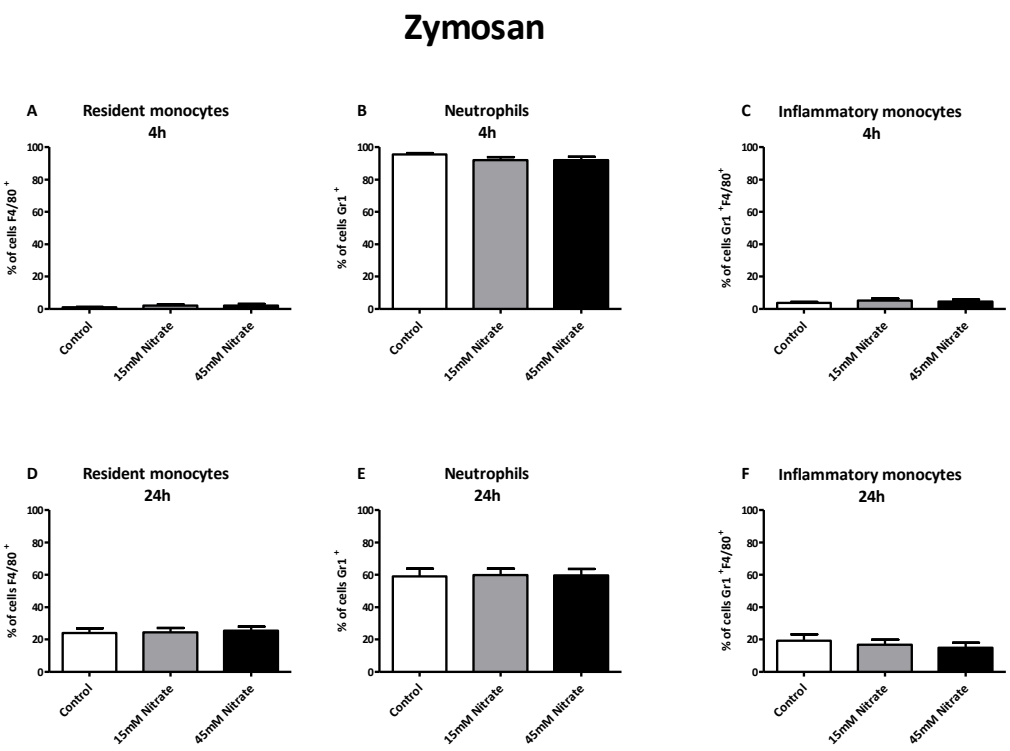


Figure 5.12: The effect of dietary nitrate on resident monocytes (A + D; n=17), neutrophils (B + E; n=17) and inflammatory monocytes (C + F; n=17) 4 and 24h after i.p. injection of zymosan (1mg). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

5.8 The effect of dietary nitrate treatment on MPO levels in the cell pellet following i.p. injection of inflammatory stimuli

Dietary nitrate supplementation had no effect on MPO levels in PBS stimulated mice 4 (figure 5.13A) and 24h (figure 5.13B) after injection.

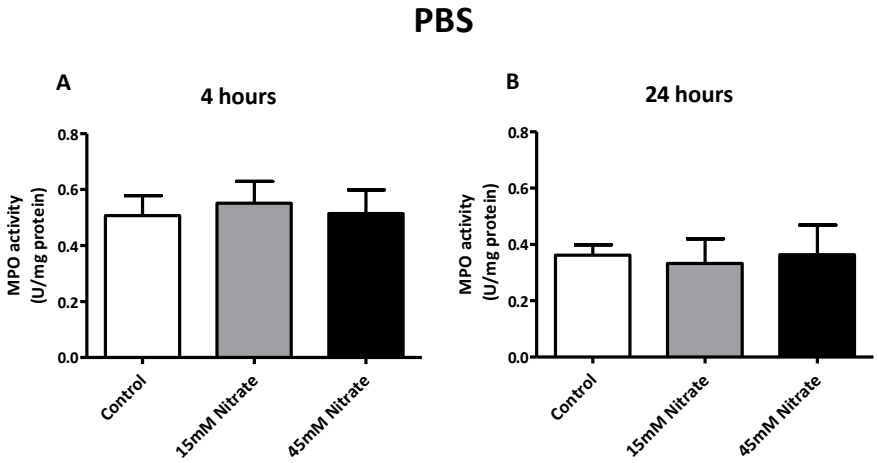


Figure 5.13: The effect of dietary nitrate on MPO activity 4 (A; n=6) and 24h (B; n=6) after i.p. injection of PBS (0.5ml). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Dietary nitrate treatment had no effect on MPO levels in IL-1β stimulated mice 4 (figure 5.14A) and 24h (figure 5.14B) after injection.

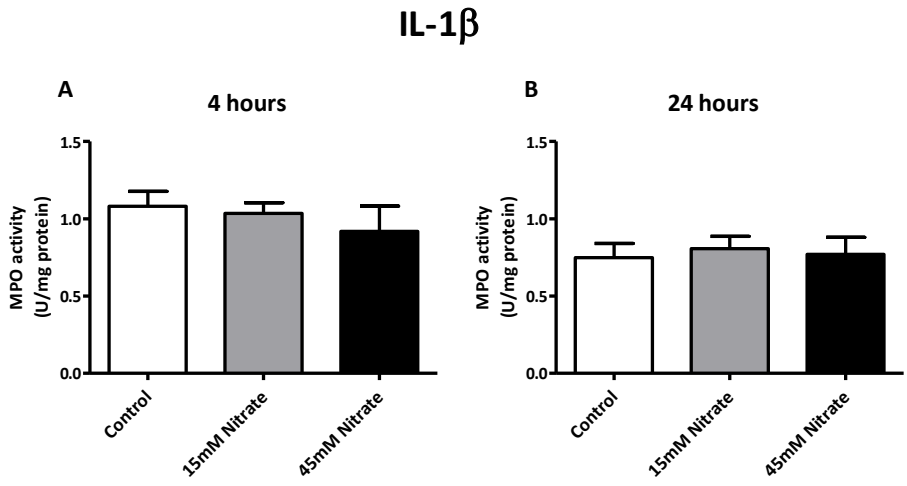


Figure 5.14: The effect of dietary nitrate on MPO activity 4 (A; n=6) and 24h (B; n=6) after i.p. injection of IL-1β (5ng). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Dietary nitrate supplementation caused dose-dependent decreases in MPO levels 4h after the injection of TNFα with significance being reached at the 45mM nitrate dose (figure 5.15A). This effect of nitrate was still evident 24h after injection (figure 5.15B).

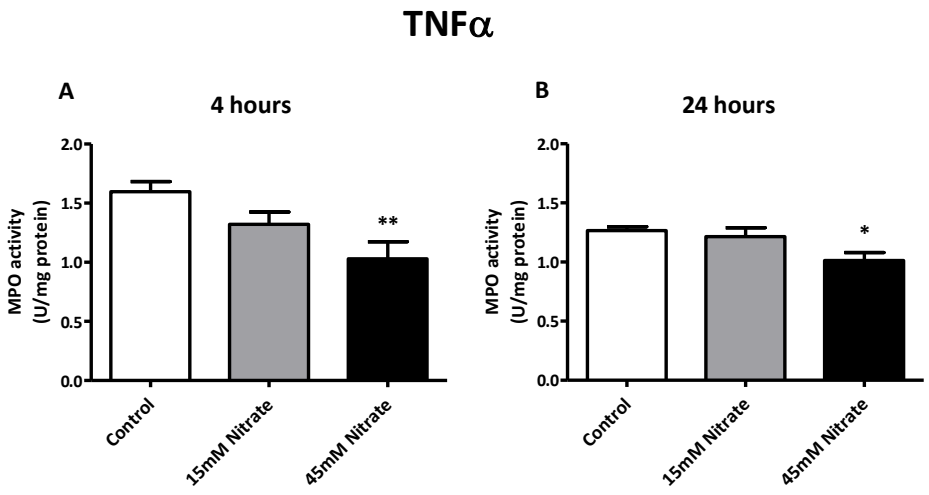


Figure 5.15: The effect of dietary nitrate on MPO activity 4 (A; n=5-6) and 24h (B; n=6) after i.p. injection of TNFα (300ng). Data shown as mean±SEM. A significant difference is shown as **p<0.01 or *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison test.

Dietary nitrate caused dose-dependent decreases in MPO levels 4h after the injection of zymosan with significance being reached at both nitrate doses (figure 5.16A). This effect of nitrate was still evident 24 after injection (figure 5.16B).

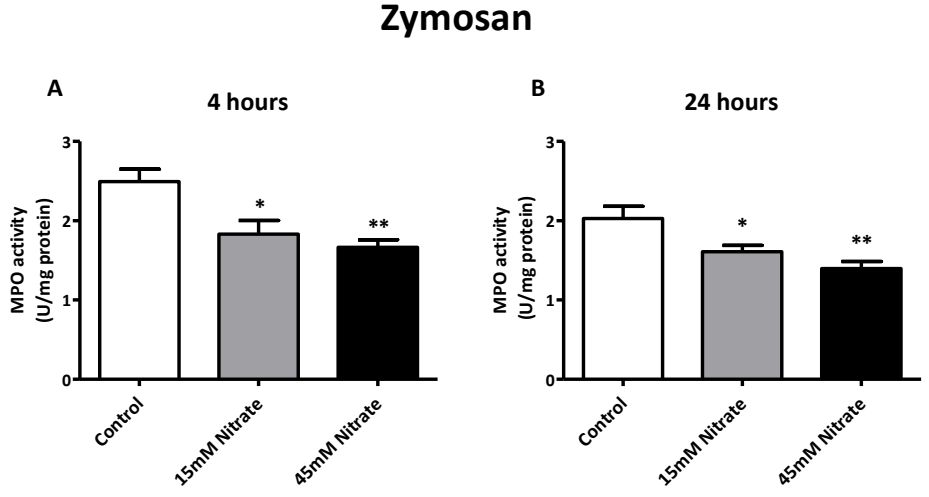


Figure 5.16: The effect of dietary nitrate on MPO activity 4 (A; n=6) and 24h (B; n=6) after i.p. injection of zymosan (1mg). Data shown as mean±SEM. A significant difference is shown as **p<0.01 or *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison test.

5.9 The effect of dietary nitrate treatment on MPO levels in the mesentery following i.p. injection of inflammatory stimuli

Dietary nitrate supplementation had no effect on MPO activity in PBS injected mice 4 (figure 5.17A) and 24h (figure 5.17B) after the injection of stimulus.

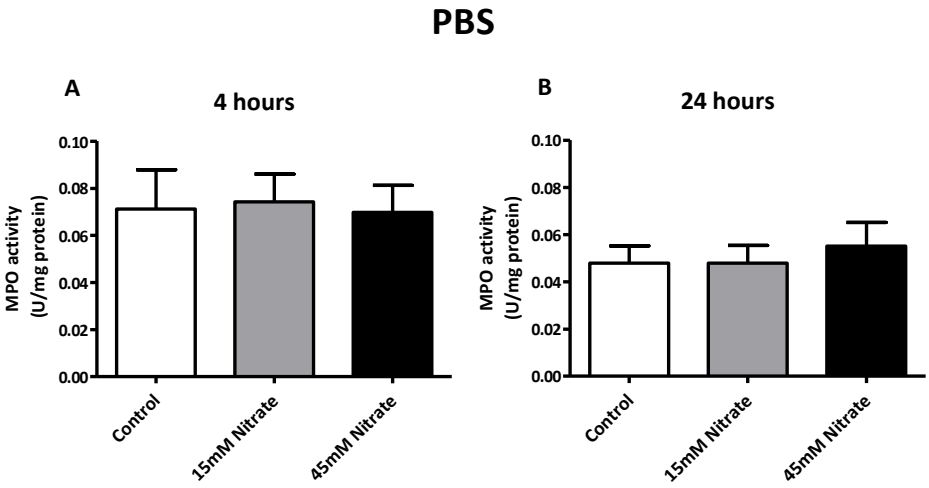


Figure 5.17: The effect of dietary nitrate on MPO activity 4 (A; n=8-9) and 24h (B; n=6) after i.p. injection of PBS (0.5ml). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Similarly to PBS injected mice dietary nitrate treatment had no effect on MPO activity in IL-1β stimulated mice 4 (figure 5.18A) and 24h (figure 5.18B) after injection.

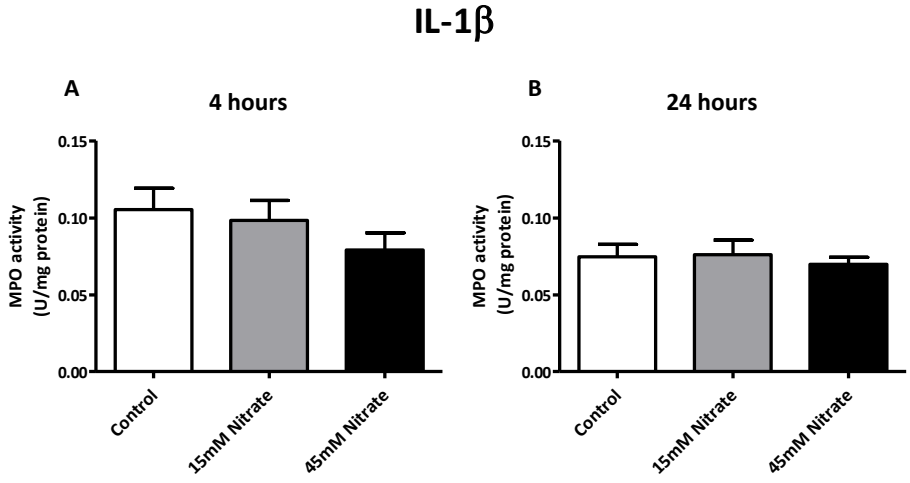


Figure 5.18: The effect of dietary nitrate on MPO activity 4 (A; n=10) and 24h (B; n=9) after i.p. injection of IL-1β (5ng). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Interestingly dietary nitrate supplementation caused dose-dependent decreases in MPO activity 4h after the injection of TNFα with significance being reached at both doses of nitrate (figure 5.19A). This effect of nitrate was not apparent 24h after the injection of stimulus as no effect of dietary nitrate on MPO level was observed after 24h (figure 5.19B).

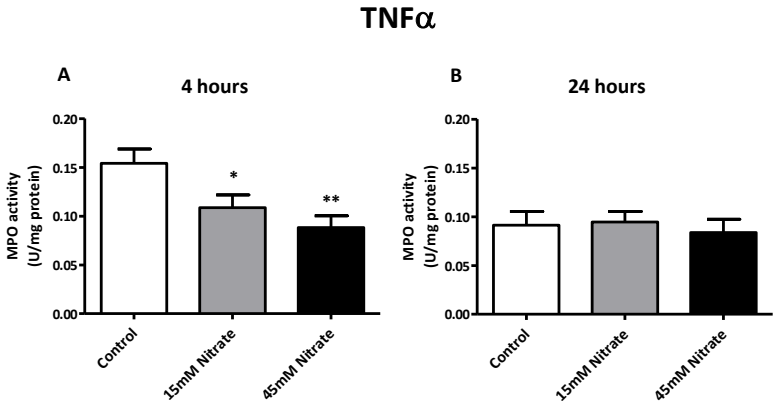


Figure 5.19: The effect of dietary nitrate on MPO activity 4 (A; n=10) and 24h (B; n=10) after i.p. injection of TNFα (300ng). Data shown as mean±SEM. A significant difference is shown as **p<0.01 or *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison test.

Dietary nitrate caused dose-dependent decreases in MPO levels 4h after the injection of zymosan with significance being reached for both nitrate doses (figure 5.20A). This effect of nitrate was still evident 24h after injection (figure 5.20B).

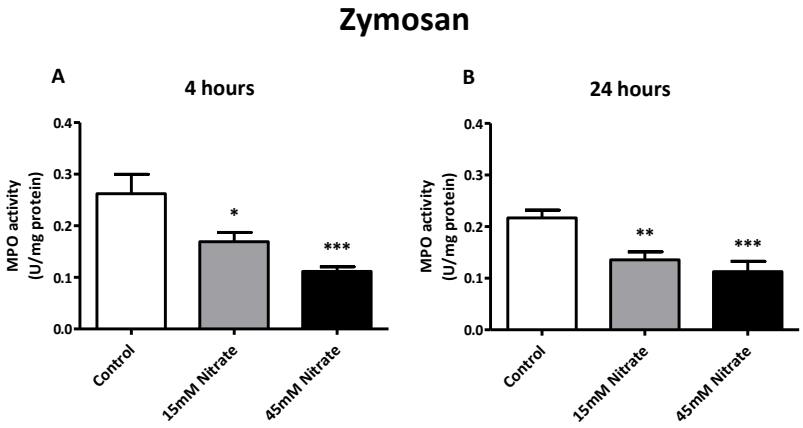


Figure 5.20: The effect of dietary nitrate on MPO activity 4 (A; n=10) and 24h (B; n=10) after i.p. injection of zymosan (1mg). Data shown as mean±SEM. A significant difference is shown as ***p<0.001, **p<0.01 or *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison test.

5.10 The effect of dietary nitrate treatment on cell surface marker expression following i.p. injection of zymosan

CD162 expression on the surface of resident monocytes (figure 5.21A + D), neutrophils (figure 5.21B + E) and inflammatory monocytes (figure 5.21C + F) was not altered by dietary nitrate supplementation when compared to control animals 4 and 24h after the injection of zymosan.

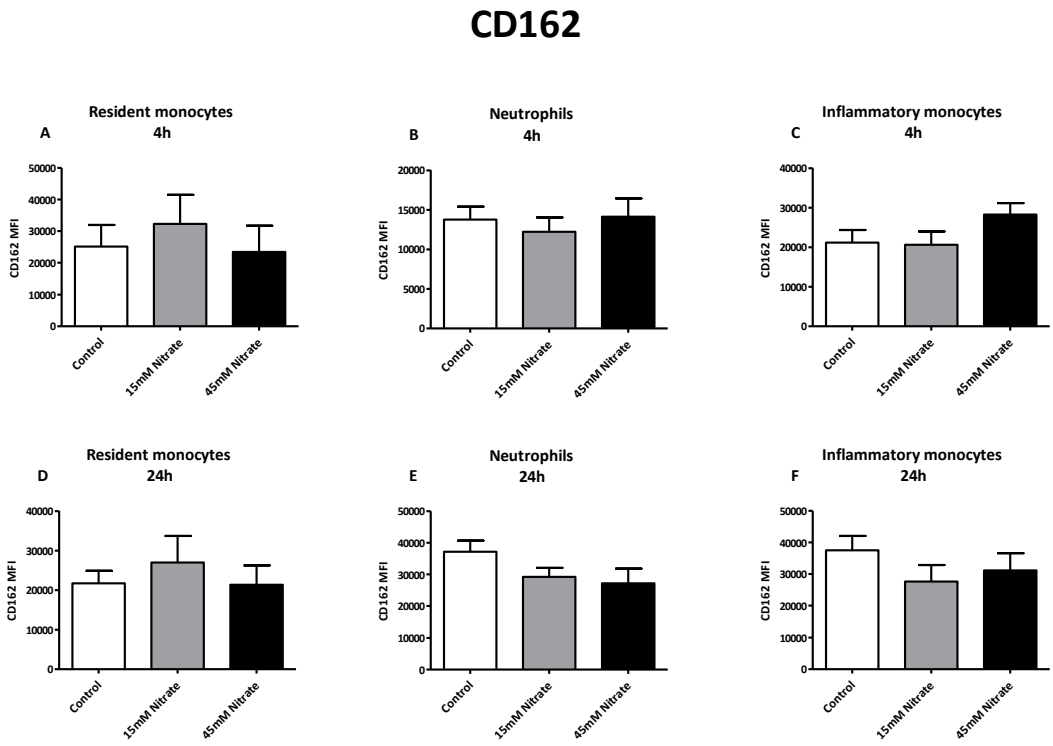


Figure 5.21: The effect of dietary nitrate on CD162 cell surface expression on resident monocytes (A + D; n=8), neutrophils (B + E; n=8) and inflammatory monocytes (C + F; n=8) 4 and 24h after i.p. injection of zymosan (1mg). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

CD62L expression on the surface of resident monocytes (figure 5.22A + D), neutrophils (figure 5.22B + E) and inflammatory monocytes (figure 5.22C + F) was not altered by a dietary nitrate treatment when compared to control animals 4 and 24h after the injection of zymosan.

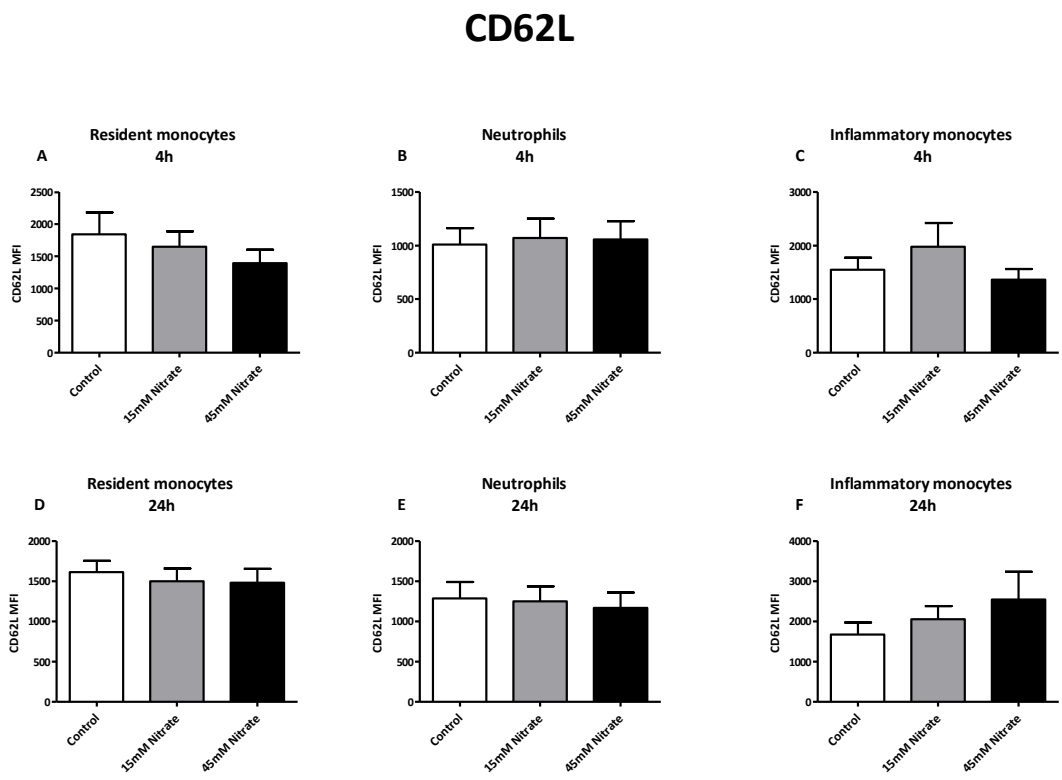


Figure 5.22: The effect of dietary nitrate on CD62L cell surface expression on resident monocytes (A + D; n=8), neutrophils (B + E; n=8) and inflammatory monocytes (C + F; n=8) 4 and 24h after i.p. injection of zymosan (1mg). Data shown as mean±SEM. Data analysed using 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

CD11b expression on the surface of resident monocytes (figure 5.23A + D) and inflammatory monocytes (figure 5.23C + F) was not altered by a dietary nitrate strategy when compared to control animals 4 and 24h after the injection of zymosan. Expression on the surface of neutrophils was dose-dependently reduced by dietary nitrate treatment 4 (figure 5.23B) and 24h (figure 5.23E) after injection.

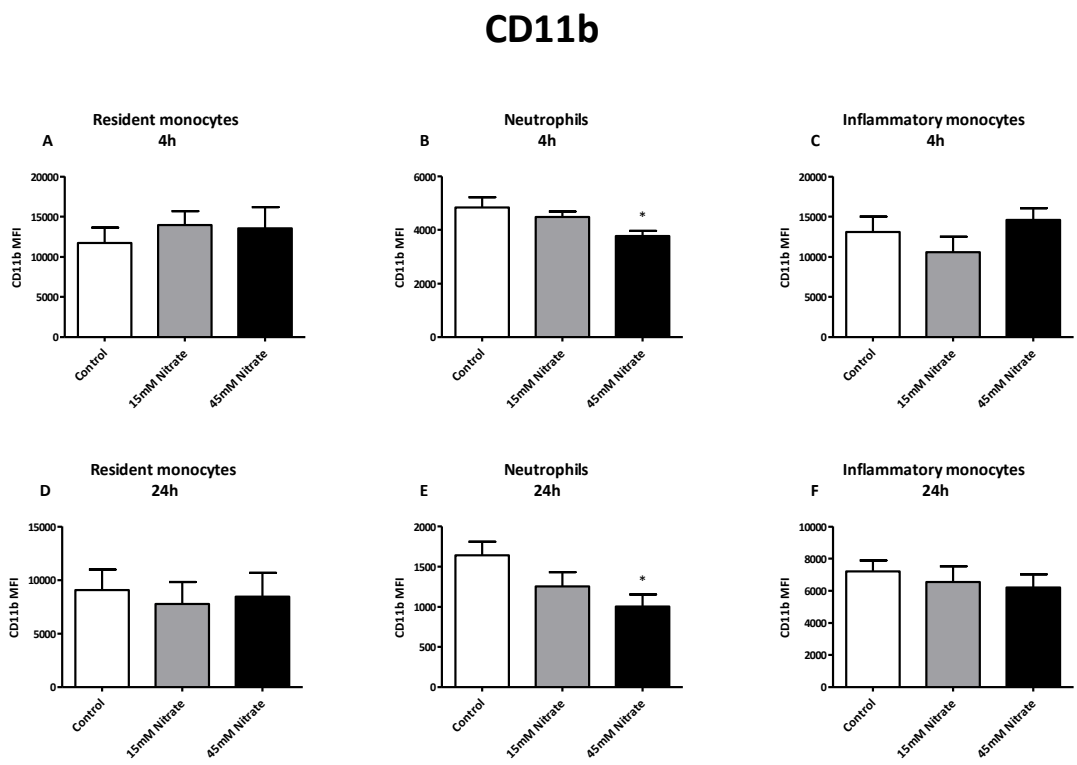


Figure 5.23: The effect of dietary nitrate on CD11b cell surface expression on resident monocytes (A + D; n=8), neutrophils (B + E; n=8) and inflammatory monocytes (C + F; n=8) 4 and 24h after i.p. injection of zymosan (1mg). Data shown as mean±SEM. A significant difference is shown as *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

5.11 The effect of dietary nitrate treatment on lavage NO_x following i.p. injection of inflammatory stimuli

Inorganic dietary nitrate supplementation caused a concentration-dependent elevation of nitrate levels in the lavage fluid collected from PBS (figure 5.24A), IL-1β (figure 5.24B), TNFα (figure 5.24C) and zymosan (figure 5.24D) treated mice 4h after injection.

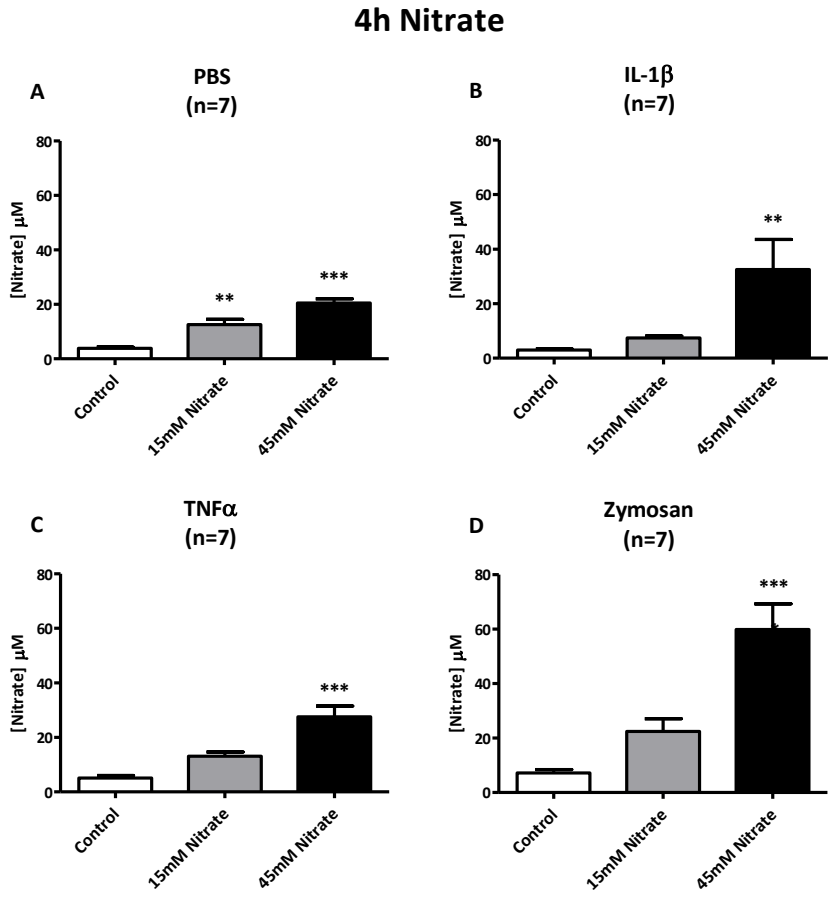


Figure 5.24: A comparison of the effect of dietary nitrate supplementation on lavage nitrate concentration with PBS (A), IL-1β (B), TNFα (C) and zymosan (D) injected mice 4h after i.p. injection of stimulus. Data shown as mean±SEM with n indicated on graphs. A significant difference is shown as ***p<0.001 or **p<0.01 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Inorganic dietary nitrate supplementation caused a concentration-dependent elevation of nitrate levels in the lavage fluid of PBS (figure 5.25A), IL-1β (figure 5.25B), TNFα (figure 5.25C) and zymosan (figure 5.25D) injected mice. This was evident in the lavage fluid collected 24 hours after the induction of inflammation.

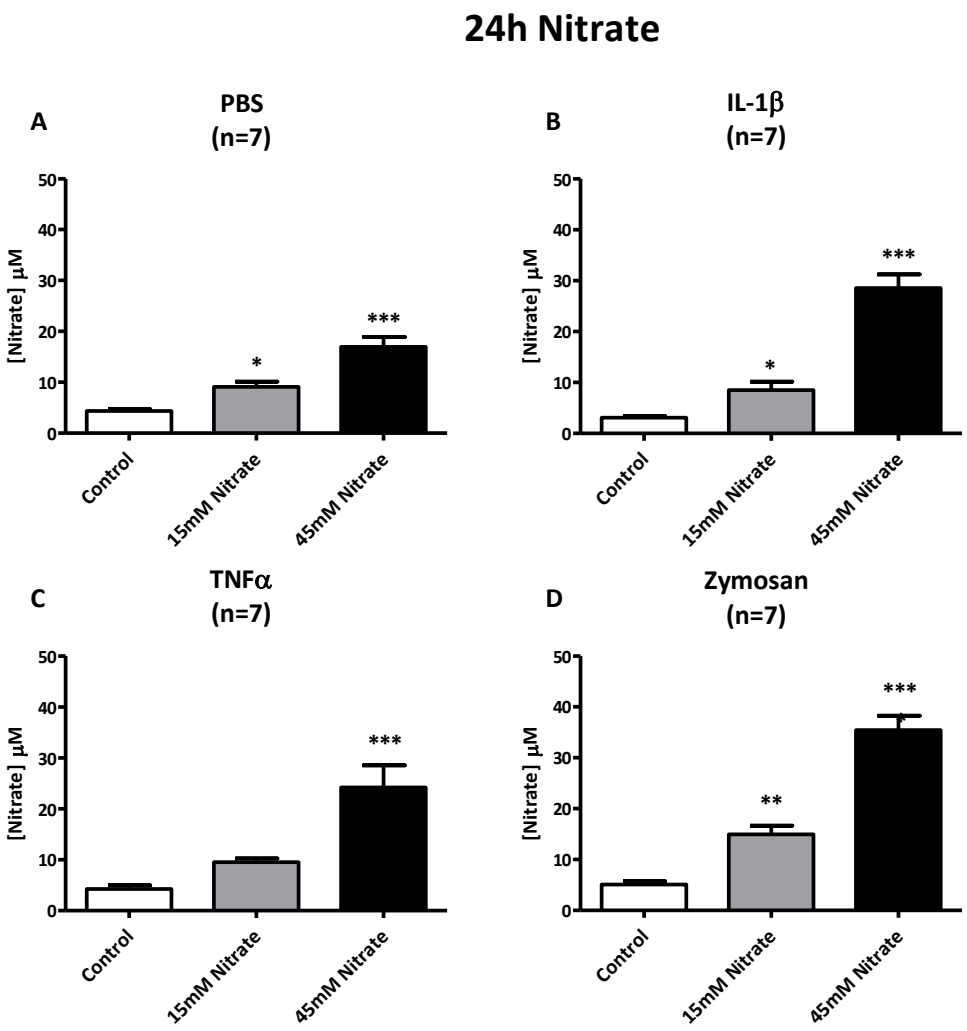


Figure 5.25: A comparison of the effect of dietary nitrate supplementation on lavage nitrate concentration with PBS (A), IL-1β (B), TNFα (C) and zymosan (D) injected mice 24h after i.p. injection of stimulus. Data shown as mean±SEM with n indicated on graphs. A significant difference is shown as ***p<0.001, **p<0.01 or *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Inorganic dietary nitrate supplementation caused a concentration-dependent elevation of nitrite concentration in the lavage fluid collected from PBS injected mice (figure 5.26A). This elevation was also shown in IL-1β (figure 5.26B), TNFα (figure 5.26C) and zymosan (figure 5.26D) stimulated animals with significance being reached at the 45mM dose for all stimuli.

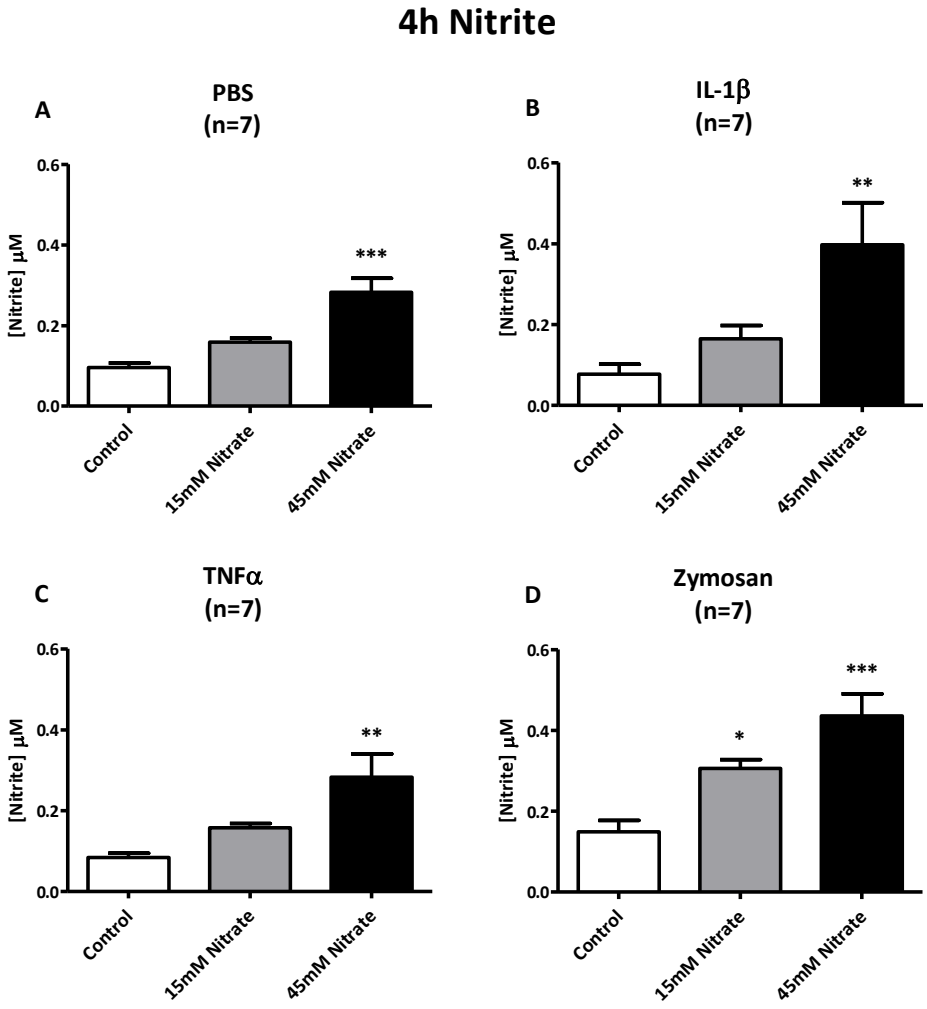


Figure 5.26: A comparison of the effect of dietary nitrate supplementation on lavage nitrate concentration with PBS (A), IL-1β (B), TNFα (C) and zymosan (D) injected mice 4h after i.p. injection of stimulus. Data shown as mean±SEM with n indicated on graphs. A significant difference is shown as ***p<0.001, **p<0.01 or *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

Inorganic dietary nitrate supplementation caused a concentration-dependent elevation of nitrite concentration in the lavage fluid collected from PBS (figure 5.27A), IL-1β (figure 5.27B), TNFα (figure 5.27C) and zymosan (figure 5.27D) injected mice. This was evident in the lavage fluid collected 24h after the induction of inflammation.

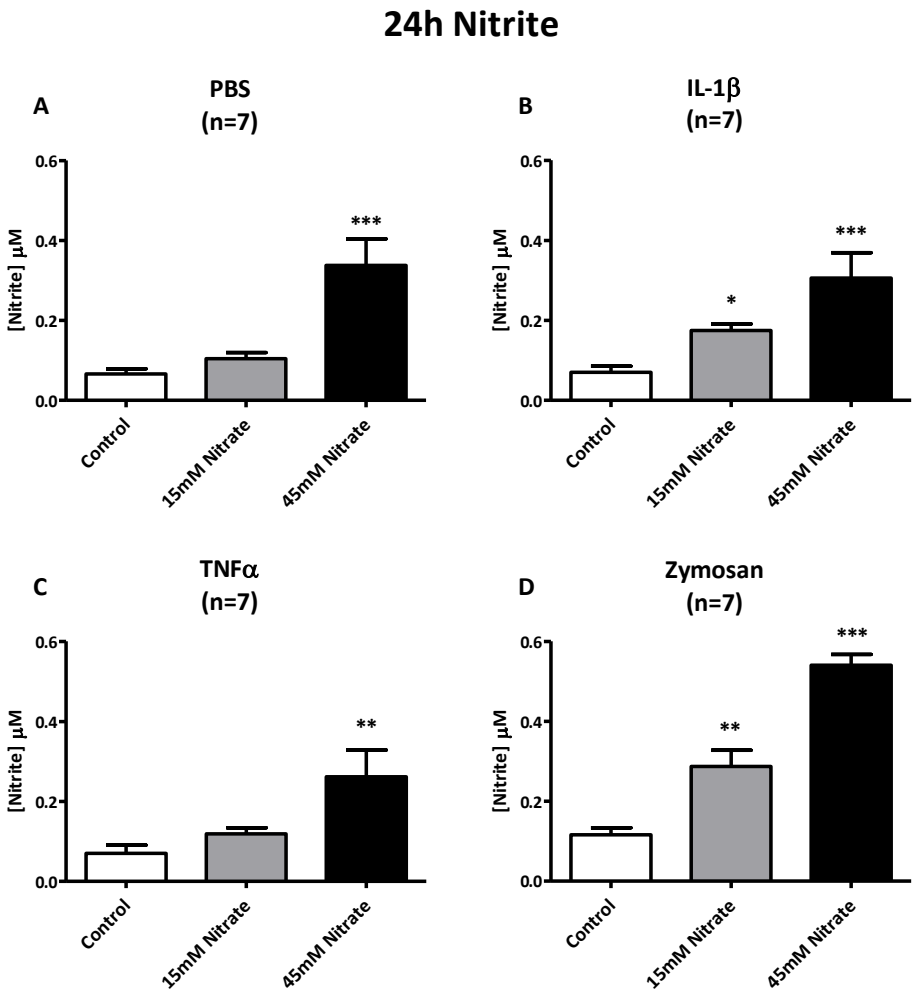


Figure 5.27: A comparison of the effect of dietary nitrate supplementation on lavage nitrite concentration with PBS (A), IL-1β (B), TNFα (C) and zymosan (D) injected mice 24h after i.p. injection of stimulus. Data shown as mean±SEM with n indicated on graphs. A significant difference is shown as ***p<0.001, **p<0.01 or *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

5.12 The effect of dietary nitrate treatment on plasma NO_x following i.p. injection of inflammatory stimuli

In WT mice inorganic dietary nitrate supplementation caused concentration-dependent elevations of plasma nitrate in PBS injected mice (figure 5.28A). This elevation was also shown in IL-1β (figure 5.28B), TNFα (figure 5.28C) and zymosan (figure 5.28D) stimulated mice.

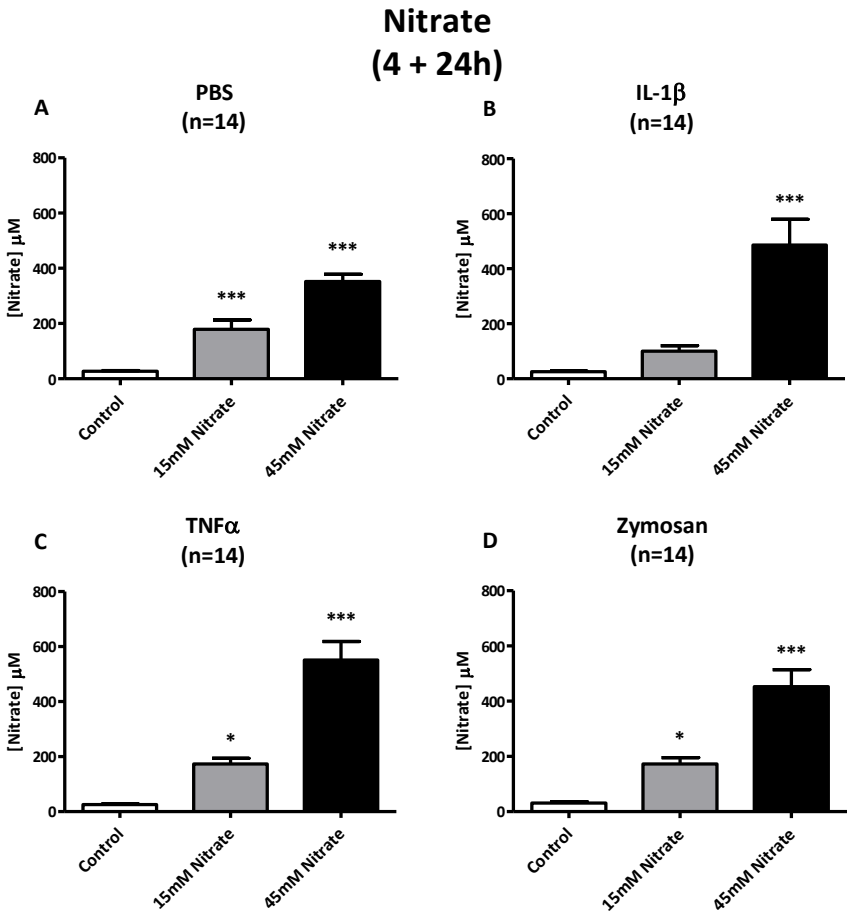


Figure 5.28: A comparison of the effect of dietary nitrate supplementation on plasma nitrate with PBS (A), IL-1β (B), TNFα (C) and zymosan (D) injected mice. Data shown as mean±SEM with n indicated on graphs. A significant difference is shown as ***p<0.001 or *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

In WT mice inorganic dietary nitrate supplementation caused concentration-dependent elevations of plasma nitrite in PBS injected mice (figure 5.29A). This elevation was also shown in IL-1β (figure 5.29B), TNFα (figure 5.29C) and zymosan (figure 5.29D) stimulated mice.

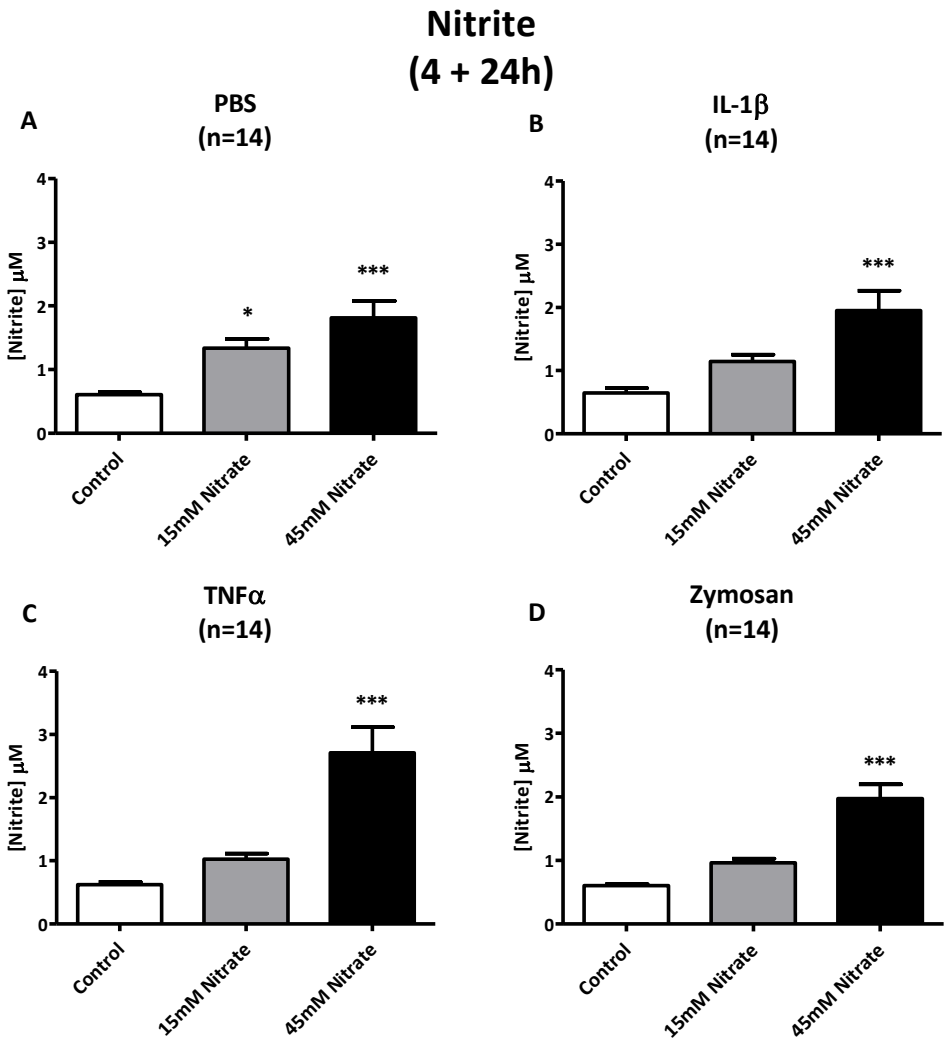


Figure 5.29: A comparison of the effect of dietary nitrate supplementation on plasma nitrite with PBS (A), IL-1β (B), TNFα (C) and zymosan (D) injected mice. Data shown as mean±SEM with n indicated on graphs. A significant difference is shown as ***p<0.001 or *p<0.05 vs. control group using a 1-way ANOVA followed by Dunnett's multiple comparison statistical test.

5.13 Summary

In this chapter it was investigated whether dietary nitrate could affect inflammatory cell recruitment using the mouse peritonitis model of acute inflammation. Peritonitis was induced by various proinflammatory cytokines and the effect of dietary nitrate on markers of inflammation was investigated. In PBS-injected WT mice dietary nitrate (both 15 and 45mM doses) significantly elevated plasma nitrate and nitrite levels above baseline. This was applicable for all stimuli used. Elevations in plasma NO_x were paralled with increases in lavage fluid NO_x.

After 4 hours, percent cell type following PBS injection was predominantly resident monocytes (93.79±3.06%) with few inflammatory monocytes (3.09±1.53%) and neutrophils (3.11±1.54%). Treatment with IL-1β elevated percentage of inflammatory monocytes (15.96±2.74%) and neutrophils (19.42±3.38%) 4 hours after injection. Treatment with TNFα resulted in an even greater percentage of inflammatory monocytes (21.29±1.72%) and neutrophils (44.13±5.84%) 4 hours after injection. Injection with zymosan caused the greatest percentage change in neutrophils (95.43±0.95%). After 24 hours IL-1β and TNFα had cell percent profiles that were similar to PBS, however zymosan still had a significant percentage of neutrophils (59.10±4.60%) present. For all stimuli dietary nitrate supplementation had no effect on percent cell type. Interestingly dietary nitrate supplementation did cause concentration-dependent reductions in total leukocyte count in TNFα (4 hours) and zymosan (4 and 24 hours) treated mice. The concentration-dependent reductions were also present in MPO levels assessed in the peritoneal infiltrate and mesentery.

Finally, in zymosan treated mice, repression of neutrophil recruitment was associated with concentration-dependent decreases in the expression of the neutrophil activation marker CD11b, at both 4 and 24 hours. These findings suggest that inorganic dietary nitrate supplementation suppresses the acute inflammatory response.

Chapter 6:

Discussion

The nitrate-nitrite-NO pathway has emerged as an alternative to the L-arginine-NOS pathway for the generation of NO. Bioactivation of nitrate occurs through its initial reduction to nitrite, a process catalysed by commensal bacteria in the mouth and GI tract (Lundberg *et al.*, 2004). Subsequently, nitrite is then further metabolised to NO and other nitrogen oxides in blood and tissues (Lundberg *et al.*, 2008). Interest in this alternative pathway stems from the view that it may provide an endogenous source of NO when defects in the production or activity of the conventional NO pathway due to endothelial dysfunction are present. This is important since the healthy endothelium exerts a number of vasoprotective effects such as vasodilation, suppression of smooth muscle cell growth and inhibition of inflammatory responses (Moncada *et al.*, 1991).

6.1 The distribution of nitrite and nitrate in health and models of CVD

6.1.1 Baseline levels of nitrite in the plasma in health

In healthy WT mice that were fed nitrite/nitrate-free water for 2 weeks plasma nitrite concentration was 0.56±0.10μM. These results are similar to previous work utilising similarly to this study low nitrite and nitrate-deplete water. Using 3 different analytical procedures, in 2003 Kleinbongard et al. determined basal plasma nitrite concentration in C57BL/6J mice to be 0.46±0.05μM (Kleinbongard *et al.*, 2003). This level is also similar to the 0.29±0.05μM and 0.40μM concentrations found in previous work (Bryan *et al.*, 2004; Stokes *et al.*, 2009). Indeed, a relatively recent review comparing plasma nitrite concentrations across studies indicated that the vast majority of measurements estimate baseline plasma nitrite concentration, in healthy volunteers, to fall within 0.20-0.50μM range (Grau *et al.*, 2007a). However, published values range from non-detectable or as low as ~0.02μM (Pannala *et al.*, 2003a) to as high as 5μM (Moshage *et al.*, 1995a). Such variability may reflect true levels in plasma but are also likely to reflect differences in techniques used for measurement of nitrite; an issue debated in several reviews (Pelletier *et al.*, 2006b; Grau *et al.*, 2007a; Tsikas, 2007b).

6.1.2 The concentrations of nitrite used for nitrite supplementation

Administration of inorganic nitrite has been proposed as an effective approach to elevate circulating concentrations of nitrite in eNOS KO mice (Bryan *et al.*, 2008),

however whether it might be as effective in CVD is unknown. In chapter 3 I determined the effects of nitrite (and nitrate) administration as a potential strategy to elevate circulating and/or tissue nitrite levels in mice. I did this first in healthy mice to ascertain a dosing regimen in mice that would provide a rise in circulating nitrite levels similar to the levels achieved in humans that have been proposed to be bioactive, for future use in functional experiments.

Animals were treated with 0.6 and 1.0mM nitrite in the drinking water. The 0.6mM nitrite concentration was chosen based on previous work where protective effects of this dose have been shown. Supplementation in the drinking water for 7 days elevated plasma levels of nitrite and this was associated with significantly reduced infarct size after myocardial I/R injury (Bryan *et al.*, 2007). The 1.0mM nitrite dose was chosen to investigate whether a dose-dependent change could be seen. Previous work has shown that nitrite given intravenously immediately before reperfusion can protect from I/R injury (Duranski *et al.*, 2005) but due to the relatively short circulating half-life of nitrite in blood (110 seconds) (Kelm, 1999), it was previously unclear as to whether nitrite administered subchronically in the drinking water could affect blood and tissue nitrite concentrations.

Nitrite in the diet serves a multitude of purposes such as fixing meat colour, contributing to the cured meat flavour and inhibiting the growth of microorganisms (specifically Clostridium botulinum) on foods. The concentrations chosen in this study equate to doses ingested in the mice of 7.00±2.00 and 13.56±0.43mg/kg/day. The main source of nitrite in the diet in western societies is processed meat and for example the amount obtained from cured, cooked sausage on average is 7.6mg/kg/day (Sindelar *et al.*, 2012). Thus the doses administered in this study reflect those consumed in the diets of humans.

6.1.3 Dietary nitrite supplementation elevates plasma nitrite

These concentrations of nitrite (0.6 and 1.0mM) in the drinking water resulted in concentration-dependent rises of nitrite in the plasma of 1.50- and 1.76-fold

respectively. Assessment of basal levels of nitrite in the tissues indicated that uptake of nitrite was greatest in the aorta. Indeed the aorta had ~20-fold higher levels of nitrite compared to the kidney, heart and liver and ~9-fold higher compared to the lung. The aorta, being a conduit vessel has a primary function of transportation therefore the difference in nitrite levels could be attributed to increased consumption by the other tissues. Indeed in 2004, Bryan et al. showed a similar disparity in nitrite levels found in the aorta compared to other organs including the kidney, heart, liver and lung (Bryan *et al.*, 2004). The dosing regimen had a similar effect throughout the cardiovascular system causing concentration-dependent increases in nitrite levels for all organs tested. Indeed the fold elevations seen in all organs were similar to the fold elevations to those observed in the plasma.

Baseline tissue concentrations of nitrite tend to be greater than those measured in plasma. Recent studies analysing nitrite levels in various organs in the rat measured the highest concentrations of nitrite in the vasculature (~12-23µM) in comparison with most other tissues; levels fell between 0.2-0.5µM in the heart, brain, liver, kidney and lung (Bryan *et al.*, 2004; Bryan *et al.*, 2005), a similar difference observed in my data. Variation in the literature does exist e.g. in the rat heart levels of up to 10µM have been measured (Zweier *et al.*, 1995) although these may be due to differences in dietary intake, rather than reflecting differences in endogenous production, or may simply be a reflection of differences in techniques of measurement.

Previous studies investigating distribution of nitrite stores in the rat have shown that supplementation of nitrite by systemic injection results in a rapid (steady-state concentrations achieved within 5 min) distribution throughout the body (Bryan *et al.*, 2005). However, feeding animals a low nitrate diet resulted in substantial decreases in tissue levels throughout the body with the notable exception of plasma and aorta, where levels were maintained at those observed in animals fed a standard chow diet. The authors suggested that these observations, taken together, intimate the existence of 2 distinct processes involved in nitrite distribution. Acute elevation results in passive transport of nitrite across membranes while, in contrast, slow changes in plasma

nitrite are sensed by the NOS within circulating cells and the vascular wall, the activity of which alters to maintain basal levels (Bryan *et al.*, 2005).

With regard to the distribution of nitrite throughout the organs, questions still remain as to the mechanism of uptake into the cells since the charged nature of nitrite has prompted speculation that specific uptake mechanisms for nitrite must exist or that nitrite utlilises anion exchangers to enter cells. Studies using equine RBC ghost demonstrated nitrite exchange (export) with bicarbonate in the place of chloride via the anion exchange transporter-1 (AE-1), since it was blocked by the non-selective inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulphonate (Shingles *et al.*, 1997). It has also been suggested that the Na-P_i transporter plays a role in nitrite uptake in RBCs, since addition of phosphate and removal of sodium, to the extracellular media bathing isolated human RBCs, inhibited nitrite uptake by ~25% (May *et al.*, 2000).

In eukaryotic cells it has been proposed that conversion of nitrite to nitrous acid (HNO_2) underlies nitrite entry into cells, however with a pK_a of 3.3-3.4 for HNO_2 a very acidic pH is required for such a phenomenon. Whilst it is likely that this pathway contributes to nitrite transport from the acidic environment of the gastric lumen, where the pH is between 1.0-2.5 (Evans *et al.*, 1988), to the circulation, it is unlikely that this accounts for substantial transport within the cells of the cardiovascular system, where the pH of the blood drops to 6.8 only under the most severe acidotic conditions (Williamson *et al.*, 1976) and perhaps to 5.5 deep within ischaemic tissues (Zweier *et al.*, 1995). Whether the pathways identified in RBCs might also have a role to play in tissue nitrite uptake is uncertain and warrants further investigation.

6.1.4 Baseline levels of nitrate in the plasma in health

In healthy mice that were fed nitrate-free water for 2 weeks baseline levels of nitrate were 37.68±2.26µM. Plasma levels of nitrate in health have been suggested to be in the 20-40µM range (Lundberg *et al.*, 2004; Webb *et al.*, 2008c; Kapil *et al.*, 2010). This is a key observation as nitrate levels have been proposed to be up to 100 times more than nitrite levels (Webb *et al.*, 2008c; Kapil *et al.*, 2010). In this study baseline plasma

nitrate concentration was measured to be ~67 fold more than baseline plasma nitrite concentration, which is in agreement with most previous work. In humans baseline plasma nitrate levels in healthy volunteers is thought to derive from dietary nitrate (75%) (Lundberg *et al.*, 2004) and endogenous NO oxidation (Moncada *et al.*, 1993; Kelm, 1999).

6.1.5 The concentration of nitrate used for nitrate supplementation

I investigated whether dietary nitrate supplementation in mice would increase steady-state plasma and tissue levels of nitrite and nitrate. The doses of nitrate were chosen based on data obtained in studies in animal models and healthy volunteers demonstrating that the sustained elevation of plasma nitrite at low micromolar concentrations is associated with functional effects of NO (Bryan *et al.*, 2008; Webb *et al.*, 2008c; Kapil *et al.*, 2010). The concentrations of nitrate in the drinking water used in this study were chosen to cover the range of nitrate concentrations used previously in these human experiments.

6.1.6 Dietary nitrate supplementation elevates plasma nitrite and nitrate

My studies show that in mice as in humans there is a concentration-dependent effect on both plasma nitrite and nitrate levels. Dietary feeding with 0.5, 15 and 45mM nitrate supplemented water led to 1.43, 2.68 and 2.75 fold increases in plasma nitrite and 1.50, 6.80 and 18.37 fold increases in plasma nitrate. This finding suggests that as in humans the enterosalivary circuit is intact. In this study I did not confirm that disruption of the oral microbiota blocked this rise, however, this has been demonstrated by others (Govoni *et al.*, 2008; Kapil *et al.*, 2010). In addition the ratios suggest that there is likely a saturation of nitrate to nitrite conversion. This might suggest that in mice the oral conversion is saturated and that uptake of nitrite in the gut is limited. The ratios of nitrite:nitrate of 0.95, 0.39 and 0.15 suggest that with increasing amounts of nitrate relatively less nitrite is formed. Interestingly 45mM nitrate only caused a marginal increase in nitrite above 15mM (2.75 vs. 2.68) and thus the 15mM concentration was chosen for all future experiments.

The dosing regimen had a similar effect throughout the cardiovascular system causing concentration-dependent increases in nitrite and nitrate levels for all organs tested. Indeed the fold elevations seen in all organs were similar to the fold elevations to those observed in the plasma. Interestingly, as demonstrated with nitrite levels, baseline nitrate uptake was most efficiently evident in the aorta, which had ~4.41 fold more nitrate than other tissues tested. This was a difference that was also previously observed by Bryan et al. who found ~10-fold more levels of nitrate in the aorta compared to other tissues (Bryan *et al.*, 2004).

6.1.7 The effect of atherosclerosis on nitrite and nitrate distribution

Similar to humans, ApoE KO mice are characterised by endothelial dysfunction caused by their propensity to spontaneously develop hypercholesterolaemia associated with atherosclerotic lesions (Meyrelles *et al.*, 2011). Although, whether this dysfunction translates to variations in circulating levels of NO_x is uncertain with evidence demonstrating increases, decreases or no effect in humans with atherosclerosis (Maejima *et al.*, 2001; Vanizor *et al.*, 2001; Pekdemir *et al.*, 2004; Loffredo *et al.*, 2007; Binh *et al.*, 2011). In my study I have shown that at baseline and in the blood compartments nitrite levels were not significantly altered compared to WT littermates, whereas nitrate levels were reduced ~50% in the ApoE KO compared to the WT mice in plasma (53.05%) and in the RBCs (48.47%). These effects on nitrite and nitrate levels at baseline were evident across the tissues as well i.e. no apparent change in nitrite but statistically significant reductions in nitrate (aorta (49.66%); kidney (29.81%); lung (42.51%); heart (61.93%); liver (61.43%)). Previous work intimates that nitrite is a better reflection of eNOS activity (Kelm *et al.*, 1999; Kleinbongard *et al.*, 2003), however the data presented in this thesis does not support this. My data suggests that as baseline nitrate levels are significantly reduced in ApoE KO mice and that perhaps nitrate is a better reflection of endothelial dysfunction since not only are nitrate levels significantly reduced across the tissues but indeed most NO is oxidised to nitrate (Moncada *et al.*, 1993).

6.1.8 The effect of dietary nitrite or nitrate supplementation in atherosclerosis

For further investigations, the 0.6mM nitrite and 15mM nitrate doses were chosen since they caused rises in circulating nitrite that were commensurate with rises associated with biological activity in healthy volunteers (Kapil *et al.*, 2010) and increasing nitrate dose further did not increase nitrite levels substantially above the 15mM nitrate dose. As shown with WT mice, in ApoE KO mice baseline plasma nitrite (0.45±0.06μM) and baseline plasma nitrate (19.99±2.80μM) concentrations were comparable to previous work in which concentrations of plasma nitrite and nitrate were measured (Bryan *et al.*, 2004; Stokes *et al.*, 2009). Administration of 0.6mM nitrite caused a 2.29-fold (compared to 1.50 fold in WT mice) increase in plasma nitrite concentration in ApoE KO mice. Furthermore, administration of 15mM nitrate caused a 5.39-fold (compared to 2.68 fold in WT mice) increase in plasma nitrite concentration. This disparity in fold elevation was also reflected in plasma nitrate concentration as administration of 0.6mM nitrite (WT vs. ApoE KO; 1.27 vs. 1.85) and 15mM nitrate (WT vs. ApoE KO; 6.80 vs. 12.90) caused greater fold elevations than those observed in WT mice. Interestingly the nitrite:nitrate ratios were almost identical (0.6mM – 1.19 vs. 1.24; 15mM nitrate – 0.39 vs. 0.42). These data suggest that not only is the enterosalivary circuit intact but both the oral conversion and gut uptake pathways are potentially upregulated in atherosclerosis. The fact that the nitrite:nitrate ratio is identical in ApoE KO mice compared to WT mice suggest that the oral conversion and gut pathways are upregulated to the same extent.

In WT and ApoE KO mice dietary nitrate supplementation significantly elevated nitrite and nitrate levels in RBCs to a similar degree. Further investigations revealed that elevation of nitrite and nitrate levels was also possible in other tissues of the cardiovascular system. In ApoE KO mice dietary nitrate supplementation was consistently a more efficient method of elevation of nitrite across the tissues. This suggests 2 important points. Firstly, in the ApoE KO model of atherosclerosis the enterosalivary circuit is intact. The similar ratios of nitrite/nitrate in WT and ApoE KO mice support this view. Secondly, the enterosalivary circuit is functioning efficiently enough that it can be utilised to elevate circulating levels of nitrite and nitrate back up

to and even past baseline to potentially replenish depleted NO. This is important as strategies that look to elevate NO generation by targeting conventional NO synthesis are inherently problematic due to the constraints imposed by endothelial dysfunction and disturbed eNOS function (Wilson *et al.*, 2007). In addition the organic nitrates, such as glyceryl trinitrate (GTN) and isosorbide mononitrate, represented the first class of NO donors to reach the clinical setting however, the utility of these drugs has been severly limited due to 2 main issues. The generation of NO from some of these drugs is associated with the concomitant generation of other reactive O_2 species (in particular O_2^-), which feeds back and inhibits the very pathways responsible for NO generation by these drugs (Mayer *et al.*, 2008). This phenomenon is thought to underlie the development of tolerance due to both the pro-oxidant effects and induction of endothelial dysfunction associated with continuous treatment (Bellisarii *et al.*, 2003). These effects explain both the mechanism of tachyphylaxis which limits their therapeutic use (Munzel *et al.*, 2005a) and perhaps partly explains the lack of efficacy of organic nitrates in large-scale clinical trials (Group, 1995).

Regular intake of nitrate-containing food, such as green leafy vegetables, may ensure that blood and tissue levels of nitrate and NO pools are maintained at a level sufficient to compensate for any disturbances in endogenous NO synthesis (Lundberg *et al.*, 2006). The dietary pathway may provide not only essential nutrients for NO production but also a rescue or protective pathway for people with CVD (Bryan, 2006).

6.1.9 The effect of hypertension on baseline levels of nitrite and nitrate

Endothelial dysfunction is a phenomenon common to hypertension as well as atherosclerosis. To investigate whether the distribution of nitrite and nitrate might also be altered in hypertension I measured the NO_x levels in plasma, RBC, kidney, heart, aorta, liver and lung from WKY rats and SHRs. My data shows that whilst plasma, kidney, heart, aorta, liver and lung levels of both nitrite and nitrate were unaltered in the SHR, RBC NO_x was substantially reduced in the SHR. The SHRs used in this study did present with hypertension as demonstrated by their hemodynamic parameters (WKY vs. SHR; SBP 119.3±2.6 vs. 154.4±3.9; DBP 74.4±3.8 vs. 101.1±2.7).

Interpretation of NO_x data is challenging because of the fact that multiple pathways for the generation and destruction of NO_x and NO exist (Lundberg *et al.*, 2008). Indeed, changes in plasma nitrite and/or nitrate concentration may reflect not only endothelial NO synthase activity (Lauer *et al.*, 2001), NO oxidation (Ignarro *et al.*, 1993), nitrate reduction (Lundberg *et al.*, 2004; Webb *et al.*, 2008c) or all 3 at once. The differences in the effect of atherosclerosis and hypertension on the nitrite and/or nitrate profiles across the tissues could relate to differences in these pathways or may be species specific.

Interestingly the baseline levels did show similar profiles in mice compared to rats in that the aorta had the highest levels of both metabolites, the heart, lung and kidney had similar levels and the liver had the lowest levels in both species. This could be a reflection as suggested earlier of the degree of consumption and utilisation of these anions by the tissues, or to differences in the uptake pathways into the different tissues.

6.2 Nitrite reductase activity in hypertension and atherosclerosis

Elevation of systemic nitrite levels has been shown to be associated with dose-dependent decreases in blood pressure in both healthy animals and in healthy volunteers, as well as in SHRs, demonstrating clear functional effects that have been attributed to NO. However the vascular nitrite reductase that accounts for these effects has as yet not been clearly established (Vleeming *et al.*, 1997a; Cosby *et al.*, 2003a; Dejam *et al.*, 2007a). Difficulty in isolating the vascular nitrite reductase stems, in part from the fact that nitrite reduction is enhanced with decreasing pH and oxygen tension (Zweier *et al.*, 1995) and that biochemical measurement of activity is limited in sensitivity. Therefore, often, measurement needs to be made in conditions that act in concert to upregulate the activity/expression of a number of nitrite reductase candidates including XOR, deoxyhaemoglobin and eNOS (Webb *et al.*, 2008a). Despite this it is important to appreciate that the blood pressure lowering effect of nitrite occurs under normal physiological conditions such as normoxia and pH 7.4. Whether an environment of CVD might alter nitrite reductase capacity is unknown and in

chapter 4 I investigated this question by measuring activity firstly in the compartment demonstrating the most consistent differences in NO_x levels in the SHR and ApoE KO, the RBC, a key site of nitrite reduction (Cosby *et al.*, 2003a), as well as in other tissues.

6.2.1 In vivo nitrite reductase activity is enhanced in CVD and mediated by XOR

Nitrite was reduced to NO by RBCs of WKY rats in a concentration-dependent manner. This activity was enhanced by reducing pH. Such a profile fits with previous studies demonstrating a dependence on pH (Zweier *et al.*, 1995; Webb *et al.*, 2004a; Webb *et al.*, 2008a). These experiments were all conducted in N_2 i.e. anoxia. Due to the limitations of the assay it is not possible to pick up NO under oxygenated conditions. In RBCs of SHRs nitrite caused concentration-dependent NO generation that was evidenced at pH 7.4 and enhanced by reducing pH. Importantly however, the nitrite reductase activity of SHR RBCs was almost double that of the WKY. Previous studies suggest that a key nitrite reductase on RBCs is XOR (Webb *et al.*, 2008a).

Indeed, incubation of RBCs with the XOR inhibitor allopurinol near-abolished the nitrite reductase activity of RBCs purified from SHRs but interestingly not WKY rats. Thus, suggesting that XOR located at the level of the RBC is a key site for nitrite reduction in hypertension. This fits with previous work in the lab, which has shown that nitrite causes dose-dependent decreases in blood pressure in SHRs with significant decreases being evident at concentrations of nitrite of approximately 1µmol/L within the circulation. At this dose (as well as at higher doses) nitrite had minimal effects on blood pressure in the normotensive strain control rats (Ghosh *et al.*, 2013). My data suggest that XOR located at the level of the RBC underlies the nitrite reduction occurring in vivo and therefore is likely associated with the blood pressure-lowering effects of nitrite.

The enhancement of XOR activity in hypertension may be attributed to an increase in the levels of RBC XOR expression as demonstrated by a doubling of levels measured by western blotting. This is in line with previous studies where XOR expression was found to be enhanced in the liver of SHR (Laakso *et al.*, 1998b; Suzuki *et al.*, 1998a). The liver

is one of the major sites for XOR synthesis releasing XOR into the circulation (Martin *et al.*, 2004) especially after periods of metabolic stress (Terada *et al.*, 1992b). This XOR is then carried to distant parts of the circulatory tree where it binds to glycosaminoglycans (GAGs) found on the surfaces of circulating cells, including RBCs, and on the endothelium. Indeed assessment of XOR levels in the livers of SHRs also demonstrated a doubling in expression relative to the WKY. Thus, it seems likely that elevated expression in the liver results in increased levels entering the circulation and that at least some of this circulating XOR binds to GAGs on the RBCs. Further experiments testing this theory would be of value. Perhaps by interfering with GAG expression one could test whether this then might result in a loss of acute bioactivity

Interestingly the results in hypertensive rats were mirrored in ApoE KO mice. In both liver tissue and RBCs nitrite reduction was enhanced in the ApoE KO mice compared to WT controls. Furthermore, the enhanced nitrite reductase activity observed in the ApoE KOs was attenuated by allopurinol. In addition, western blot analysis of both compartments revealed that XOR expression was increased in ApoE KO mice compared to WT mice thus suggesting the enhanced nitrite reductase activity in ApoE KO mice is due to increased XOR expression and activity. This finding is similar to observations in patients with atherosclerosis where both XO expression and endothelium-bound XOR activity is substantially increased (Spiekermann *et al.*, 2003a; Guzik *et al.*, 2006a).

Previous work has shown that nitrite was reduced to NO by RBCS of normotensive patients in a concentration-dependent manner. This activity was enhanced with reducing pH. These experiments were also conducted in N_2 (Webb *et al.*, 2008a). My work showed that in RBCs of hypertensive patients nitrite also caused concentration-dependent NO generation that was evidenced at pH 7.4 and enhanced by reducing pH. Importantly, however, the nitrite reductase activity was almost doubled that of the normotensive patients.

RBCs isolated from hypertensive patients also expressed nitrite reductase activity that was attenuated by XOR inhibition at pH 7.4. Previous work in the lab has

demonstrated that RBC-facilitated nitrite reduction was unaffected by XOR inhibition at physiological pH of normotensive healthy volunteers; a role for XOR only occurring under severely acidic pH (Webb *et al.*, 2008a). This work is complemented by studies in which it was demonstrated that nitrite-induced vasodilation in the forearm of healthy volunteers was unaffected by allopurinol (Dejam *et al.*, 2007a). The observations that I have made in chapter 4 suggest that XOR activity is upregulated in human hypertension similarly to the situation in the SHR and the ApoE KO mice therefore XOR does not seem to contribute to nitrite reduction in health but mediates nitrite reduction in pathological scenarios. Moreover RBC XOR expression was doubled in hypertensives in comparison with normotensives a pattern that mimics the findings in the rat model of hypertension and the mouse model of atherosclerosis. Importantly, RBC nitrite and nitrate levels were found to be significantly lower in SHR compared with WKY as well in ApoE KO compared to WT mice. This could be due to an increased consumption of nitrite basally in the SHR and ApoE KO mouse attributed to an upregulation of nitrite reductase pathways, a phenomenon proposed to underlie the arteriovenous gradient in RBC nitrite concentrations in humans (Gladwin *et al.*, 2000; Dejam *et al.*, 2005). Together, the findings suggest a CVD-specific elevation of nitrite reductase capacity, which is in part dependent on XOR. These results also suggest that the pre-clinical models of CVD are useful models to explore the pathways involved in the increased XOR expression and activity that is also evident in human disease.

6.2.2 The enhanced nitrite reductase activity in hypertension has translational potential

That nitrite and nitrite reductase activity might similarly be more potent in hypertensive patients is supported by proof-of-principle studies demonstrating that elevation of circulating nitrite, to levels previously shown to be insufficient to cause significant blood pressure-lowering in healthy volunteers (Kapil *et al.*, 2010), caused substantial reductions in blood pressure (Ghosh *et al.*, 2013). Previous work has shown that dietary nitrate lowers blood pressure in healthy volunteers (Larsen *et al.*, 2006; Webb *et al.*, 2008c; Kapil *et al.*, 2010). More recently, this observation has been extended to patients presenting with hypertension. It has now been shown that

following oral ingestion of a dietary nitrate load, the plasma levels of both nitrate and nitrite anions rise in stage 1 hypertensive patients (Ghosh *et al.*, 2013). As demonstrated previously in healthy volunteers, the rise in plasma nitrite in the patients is slow-developing with the peak effect occurring 4h post nitrate consumption. This time-lag (relative to the very rapid (30min) appearance of nitrate) reflects the dependence upon the enterosalivary circuit for the conversion of nitrate to nitrite in the body. The patient studies showed that the time-course for the bioactivation and appearance within the circulation of nitrite and nitrate is similar between normotensive and stage 1 hypertensives (Kapil *et al.*, 2010; Ghosh *et al.*, 2013). However, this study also demonstrated an important increased potency of nitrate in patients. Previous studies have shown that oral inorganic nitrate ingestion (4-24mmol in the form of a potassium nitrate capsule) causes a dose-dependent rise in plasma nitrite levels and decreases in blood pressure in healthy volunteers (Kapil *et al.*, 2010) with a 4mmol dose of nitrate representing a threshold dose for blood pressure lowering, and causing a 1.4-fold peak increase (from baseline) of plasma nitrite. Of particular significance in the hypertension patient study is that the dose administered to the patient was of approximately 3.3mmol. This lead to a peak fold increase of plasma nitrite of 1.5±0.1-fold suggesting similar processing of nitrate to nitrite in hypertensives, although a prospective study comparing age-matched normotensives and hypertensives is needed to confirm this. However, despite this the rise in nitrite resulted in a ≥12mmHg reduction in systolic blood pressure. With regards to the processing of nitrate the levels follow a similar pattern between normotensives and hypertensives. This similarity indicates that hypertension per se does not result in substantial changes in the bioconversion rate and extent of nitrate processing via the enterosalivary circuit but that the extent of nitrite reduction to NO, as reflected by the RBC activity is enhanced.

The potential importance of these data is considerable particularly when one considers that hypertension is associated with endothelial dysfunction, which is characterised by decreased bioavailability of eNOS-derived NO (Brunner *et al.*, 2005). In hypertensives, decreased levels of both nitrite and nitrate in the circulation and urine are thought to

reflect endothelial dysfunction and reduced NO synthesis (Brunner *et al.*, 2005). The findings with dietary nitrate suggest that interventions targeting NO generation via the nitrate-nitrite-NO generating pathway remain intact and can be utlilised to correct NO levels in hypertension. My data complement these findings as they indicate that the nitrite reductase pathways that facilitate the reduction of nitrite to NO are enhanced in hypertension leading to the increased potency of nitrite in hypertension and possibly other CVDs. Whether my observations in ApoE KO mice might translate to patients with atherosclerosis is uncertain. However, recent assessment of this possibility has been investigated with the completion of a randomised placebo controlled double blind clinical trial in which 67 hypercholesterolaemics were randomised to receive nitrate-deplete beetroot juice or nitrate-rich beetroot juice. Measurements of platelet and endothelial function were conducted before and after 6 weeks of juice ingestion, and publication of the findings are eagerly awaited (ClinicalTrials.gov Identifier: NCT01493752)

The benefits of a high nitrate i.e. fruit and vegetable rich diets, in populations at risk of CVD are well recognised. However, investigation of the constituent elements of such diets has not replicated the same beneficial outcomes (Bjelakovic *et al.*, 2007; Bjelakovic *et al.*, 2012). The enhanced nitrite reductase activity in CVD however, demonstrates that a dietary nitrate load at a dose that is safe and achievable, sufficiently elevating plasma nitrite levels does cause significant resultant reductions in blood pressure. It is important to note that the dietary approaches to stop hypertension (DASH) diet, which has been shown to lower blood pressure (Appel *et al.*, 1997), is thought to deliver ~20mmol of nitrate (Hord *et al.*, 2009). Importantly in a 70kg adult this would equate to ~4.2mmol. This dose exceeds the acceptable daily intake limit for nitrate, which currently is 3.7mg/kg/day (EFSA, 2008). However, the dietary nitrate load (~3.3mmol) given in the hypertensive cohort mentioned (Ghosh *et al.*, 2013) sits below this level and thus suggests that the blood pressure lowering effects of fruit and vegetable rich diets might be best achieved by consuming nitrate-rich vegetables. Given that ~50% of treated hypertensive subjects fail to achieve their target blood pressure (Egan *et al.*, 2010), an additional strategy based on nitrate-rich

foods i.e. green leafy vegetables, may prove to be both cost-effective and favourable for public health. Thus further elucidation of the mechanisms involved and the long term therapeutic potential of inorganic dietary nitrate is warranted. Indeed a clinical trial assessing a sustained once daily dietary nitrate load for 4 weeks in hypertensive patients (ClinicalTrials.gov Identifier: NCT01405898) has just been completed and again the results eagerly awaited.

6.3 Investigating the effect of dietary nitrate on inflammatory cell recruitment

Recruitment of immune cells from the circulation to tissues is fundamental for host defence against pathogens. Increases in cell activation contribute to many chronic diseases including atherosclerosis and hypertension (Hansson *et al.*, 2006). Indeed such disorders are associated with sustained low-grade inflammation, thought to be critical in disease progression (Mocsai, 2013). Among various important functions the healthy vascular endothelium maintains a leukocyte-free surface, which is necessary for the preservation of vascular homeostasis. NO is one of the most important humoral mediators produced by the endothelium (Moncada *et al.*, 1991) and influences vascular integrity by preventing adherence of platelets and leukocytes e.g. neutrophils, to the vascular endothelium (Kubes *et al.*, 1991; Gauthier *et al.*, 1995). This relationship between NO and cell adhesion molecule expression therefore has important implications for health and disease (Freedman *et al.*, 1996). One can see that supplementation of NO under circumstances in which there is insufficient NO production, such as those seen in CVD, might have therapeutic utility particularly in the modulation of leukocyte recruitment. Therefore in chapter 5 I investigated whether dietary inorganic nitrate might influence leukocyte recruitment.

6.3.1 Proinflammatory cytokines IL-1β, TNFα and zymosan effectively stimulate cell recruitment into the peritoneal cavity

Initial investigations set out to determine the effect of inflammatory stimuli on cell recruitment. In terms of total leukocyte count and in comparison to PBS (vehicle) control ($5.67\pm0.26\times10^6$ cells), i.p. injection with IL-1β ($6.20\pm0.42\times10^6$ cells) or TNFα ($6.92\pm0.73\times10^6$ cells) caused recruitment of more leukocytes into the peritoneal cavity. However zymosan ($32.91\pm2.26\times10^6$ cells) caused a particularly abundant number of

cells to be recruited. At 24h following PBS injection resolution of inflammation was evident from the total leukocyte count ($4.72\pm0.23x10^6$). Furthermore, at 24h following injection, IL-1β- ($5.97\pm0.26x10^6$ cells), TNFα- ($6.06\pm0.58x10^6$ cells) and zymosan-induced ($25.96\pm1.92x10^6$ cells) leukocyte infiltration appeared also to be undergoing resolution of the inflammation as evident from the total leukocyte count.

Interestingly, flow cytometric analysis indicated that after 4h IL-1β and TNFα caused predominantly neutrophils to be recruited, 30-40% with 20-30% of the peritoneal cells being inflammatory monocytes. In comparison, in PBS-treated mice >95% of the cells were resident monocytes and <5% neutrophils and inflammatory monocytes. Injection of zymosan also produced a time-dependent cell accumulation into mouse peritoneal cavities which followed a typical profile of acute inflammation. In accordance with previous studies there was a large predominance of neutrophils (>90%) (Ajuebor *et al.*, 1998). With regards to the time points tested, leukocyte influx was maximal at 4h post-zymosan and this was followed by an accumulation of monocytes (resident and inflammatory), which in previous studies have been shown to peak at 24h (Getting *et al.*, 1997). Furthermore, as demonstrated by previous work (Getting *et al.*, 1997), by 24h inflammation had not fully resolved as indicated by flow cytometric analysis as well as the total leukocyte count. A similar profile was evident in this study. The differences in the cell profiles in response to the inflammatory stimuli relates to differences in the pathways activated by the respective stimuli. Zymosan activation of the complement cascade (Rawal *et al.*, 1998; Mizuno *et al.*, 2009) resulted in a predominantly neutrophil-dependent response whilst the cytokines TNFα and IL-1β triggered both neutrophil and monocyte recruitment.

The recruitment of neutrophils by all 3 proinflammatory cytokines was further substantiated by measurement of MPO in whole mesentery homogenates and in cell pellets. In the mesentery 4h after treatments, zymosan caused substantial neutrophil recruitment compared to IL-1β and TNFα. After 24h IL-1β and TNFα displayed MPO levels that had almost reverted back to PBS control levels whereas for zymosan-treated animals mesenteric MPO analysis indicated that by 24h the acute

inflammatory response had not fully resolved. These results were reflected in MPO levels analysed in the cell pellet indicating recruitment of neutrophils into the cavity itself as well as into the mesenteric vasculature.

NO has previously been shown to reduce leukocyte activity and adhesion (Kubes *et al.*, 1991; Gaboury *et al.*, 1993), however whether dietary nitrite/nitrate could similarly affect inflammatory processes is uncertain. As part of my work in chapter 5 I explored the possibility that dietary nitrate may be used to modulate vascular inflammation by delivering NO.

6.3.2 Dietary nitrate attenuates inflammatory cell recruitment

My studies indicate that a dietary nitrate strategy may be an effective way to modulate the acute inflammatory response. For the 2 most potent stimulators of cell recruitment, concentration-dependent reductions in total leukocyte count were observed. In TNFα-treated animals this was only evident at 4h whereas for zymosan-treated animals the suppressive effect of nitrate treatment was evident at 4 and 24h. Previously it has been shown that acute nitrite treatment can reduce infiltration of leukocytes in response to microvascular inflammation (Jadert *et al.*, 2012; Samal *et al.*, 2012). Furthermore, in a model of NSAID-induced intestinal injury leukocyte infiltration has been shown to be reduced in mice that were pretreated with 10mM dietary nitrate (administered in the drinking water for 1 week) (Jadert *et al.*, 2012). Treatment of mice with antiseptic mouthwash during the week of dietary nitrate pretreatment resulted in loss of nitrate-induced attenuation of leukocyte infiltration. These data along with my results provide strong evidence for the utility of the nitrate-nitrite-NO pathway in influencing leukocyte infiltration, a finding that could be of particular importance for diseases in which the inflammatory response becomes dysfunctional.

6.3.3 Dietary nitrate attenuates neutrophil-mediated acute inflammation

Interestingly mice treated with either TNFα or zymosan responded most sensitively to dietary nitrate treatment. Furthermore these mice were characterized by the most abundant (in terms of cell percent, mesenteric and cell pellet MPO) levels of neutrophil recruitment. Concentration-dependent reductions in mesenteric and cell pellet MPO

levels were seen with dietary nitrate treatment 4h post zymosan treatment. This is comparable to previous work in which control treated diclofenac-challenged mice (to mimic intestinal injury) exhibited similar levels of MPO to those seen in the control treated zymosan-challenged mice in this study (Jadert *et al.*, 2012). As observed in my study a statistically significantly lower MPO level was found in tissue from diclofenac-challenged mice pretreated with dietary nitrate compared with the control group given only diclofenac. This pattern of dietary nitrate-mediated attenuation was also extended to MPO levels analysed in the cell pellet supporting the view that nitrate specifically targets the neutrophil.

Interestingly the concentration-dependent reductions in MPO levels were also shown in the TNFα-treated mice but not the IL-1β-treated mice. Although after 4 and 24h cell numbers were comparable between the 2 stimuli (IL-11β vs. TNFα; 4h – $6.20\pm0.42x10^6$ vs. $6.92\pm0.73x10^6$; 24h – $5.97\pm0.26x10^6$ vs. $6.06\pm0.58x10^6$), as previously mentioned flow cytometric analysis showed a greater percentage of neutrophils recruited in the TNFα-treated mice compared to the IL-1β-treated mice. Furthermore quantification of MPO levels in the cell pellet revealed exactly the same observations that were noted in mesenteric MPO levels. Assessment of neutrophil recruitment demonstrated clear concentration-dependent suppression of MPO levels in the mesentery and cellular infiltrate indicating that dietary nitrate supplementation not only prevented recruitment of cells into the peritoneal cavity but also likely reduced the number of cells adhering to the vascular wall.

6.3.4 Dietary nitrate suppresses neutrophilic adhesion molecule expression

To investigate whether the altered cell recruitment evident in the peritoneal cavity might be due to a dietary nitrate-dependent suppression of a specific step in the recruitment paradigm, I measured expression of specific inflammatory adhesion molecules on the surface of the leukocytes.

Leukocyte recruitment to sites of tissue injury is a dynamic, multistep process involving leukocyte rolling, adhesion and emigration (Ley *et al.*, 2007). Initial leukocyte-

endothelial cell interactions are mediated by the selectin family of adhesion molecules. L-selectin (CD62L) is expressed constitutively on the leukocyte surface and is believed to play a pivotal role in the initial rolling of leukocytes along the endothelial layer. In my studies dietary nitrate treatment did not alter expression of this adhesion molecule. This is not surprising since as mentioned this molecule is constitutively expressed. Early leukocyte rolling in vivo is mediated by the interaction of endothelial (particularly P-selectin) and leukocyte adhesion molecules (particularly PSGL-1). P- and E-selectins are subsequently expressed in a matter of minutes and hours, respectively, to aid this process (Bevilacqua *et al.*, 1993; Springer, 1994; Tedder *et al.*, 1995). Unfortunately due to the nature of my experiments I was unable to measure endothelial P-selectin. It is likely however that P-selectin expression is a target for nitrate-derived NO since it is well established that NO, via cGMP elevation, suppresses endothelial P-selectin. Indeed recent assessment of platelet P-selectin has shown a suppressive effect of nitrate in platelets of healthy volunteers activated with adenosine diphophate (Velmurugan *et al.*, 2013). I found no effect of nitrate on PSGL-1 expression.

Firm adhesion of leukocytes to the endothelium depends predominantly on the expression of β_2-integrin (CD11b/CD18) on the leukocyte surface and ICAM-1 on the endothelial cells (von Andrian *et al.*, 1991; Bevilacqua *et al.*, 1993; Kubes *et al.*, 1996). CD11b is expressed on natural killer cells and a subset of lymphocytes in addition to neutrophils and different types of monocytes (Springer, 1990) and plays a central role in the migration of leukocytes from peripheral blood to sites of inflammation during the process of host defense (Springer, 1990; Nielsen *et al.*, 1994a). Moreover the protein contributes to firm leukocyte adhesion, not only to the endothelium via ICAM-1 but also to the underlying subendothelium and interstitial extracellular matrix by binding diverse ligands such as fibronectin, collagens and laminins (Smith *et al.*, 1989; Thompson *et al.*, 1992; Ding *et al.*, 1999).

The neutrophil, one of the first cell types emerging after an acute inflammatory stimulus, is considered to be a major player in CVD aetiology. Injury of the

microvascular endothelium causes upregulation of adhesion molecules such as P-selectin and ICAM-1, which leads to recruitment of neutrophils through interactions with PSGL-1 and CD18 respectively. Neutrophils are therefore considered one of the most important effector cells in acute inflammation. Prevention of neutrophil activation and recruitment, processes partly mediated in healthy individuals by endogenously produced NO (Kubes *et al.*, 1991; Gaboury *et al.*, 1993; Lefer *et al.*, 1999; Ignarro, 2002), is therefore desirable. My studies did show that dietary nitrate induced a neutrophil specific down-regulation of the expression of CD11b. Furthermore suppression of CD11b expression was concentration-dependent. The repressive effects of nitrite/nitrate on adhesion molecule expression have been demonstrated in other studies as well. For example, acute pretreatment with a bolus dose of nitrite (1.3mg/kg) was shown to decrease neutrophil adhesion and emigration in the mouse cremaster muscle stimulated with the neutrophil specific chemokine MIP-2. These effects however, were attributed to down regulation of ICAM-1 expression (Jadert *et al.*, 2012) although CD11b expression was not measured. In addition, supplementation with dietary nitrate was shown to reduce the number of emigrated and rolling cells in MIP-2 superfused cremaster muscle, an effect attributed to prevention of P-selectin expression (Jadert *et al.*, 2012). As demonstrated in chapter 5 the ability of dietary nitrate supplementation to manipulate adhesion molecule expression but more pertinently CD11b expression on neutrophils indicates a previously uncharacterised role for dietary nitrate in the process of firm adhesion of the neutrophil to the endothelium. Arguably more importantly it suggests the use of a potent anti-inflammatory agent that could be administered and managed through the diet and may be of considerable therapeutic benefit in the treatment of inflammatory cardiovascular disorders.

Other studies demonstrating anti-inflammatory effects of dietary nitrate and nitrite are emerging in different experimental inflammatory diseases e.g. by reducing vascular pathology (Kevil *et al.*, 2011). In addition dietary nitrite supplementation has been demonstrated to prevent inflammation induced by hypercholesterolaemia, with reduced leukocyte adhesion and emigration (Stokes *et al.*, 2009). Additionally,

Carlstrom et al. recently demonstrated potent anti-inflammatory and antioxidant effects of dietary nitrate in a rat model of chronic hypertension with concomitant renal and cardiac inflammation (Carlstrom *et al.*, 2011). Observations in inflammatory bowel disease and in DSS-induced colitis also suggest that oral therapeutic nitrite and nitrate supplementation may influence the inflammatory response (Jadert *et al.*, 2014). Along with these data, the results I have provided in chapter 5 provide support for the potential modulatory effect of a dietary nitrate strategy in inflammatory disorders. However, whether nitrate/nitrite might reduce CD11b or P-selectin in CVD models of atherosclerosis and hypertension is unknown. Assessment of the inflammatory response in ApoE KO mice and in SHR would address this issue.

Much evidence now supports the notion that leukocyte recruitment is likely to be pathogenic in atherosclerotic disease. Indeed, circulating neutrophil numbers and levels of neutrophil chemokines (Zineh *et al.*, 2008) are correlated with severity in acute coronary syndromes as well as being evident in thrombi and at sites of plaque and rupture in patients (Naruko *et al.*, 2002; Weber *et al.*, 2008; Naruko *et al.*, 2010). Furthermore a relationship between hypertension and WBC count has been demonstrated with one population-based study finding an association between elevated WBC count and incident hypertension (Shankar *et al.*, 2004). In addition, leukocytes have been identified at lesional sites in the early stages of plaque formation, and neutrophil depletion is associated with decreased atherosclerotic load in mouse models of disease (van Leeuwen *et al.*, 2008; Zernecke *et al.*, 2008; Hazen, 2010). As leukocyte recruitment has been implicated in CVD and the findings presented here suggest that leukocyte recruitment can be attenuated in acute inflammation, the effects of dietary nitrate on leukocyte recruitment may in part explain the cardioprotective effects seen with green leafy vegetables.

6.4 Conclusions

The results of this thesis offer insight into how conditions associated with depleted NO production (e.g. atherosclerosis and hypertension) affect the distribution of nitrite and nitrate in the tissues. In most tissues it was observed that nitrate levels were

significantly reduced in disease models associated with cardiovascular inflammation. Moreover, my studies show that an efficient method of elevating nitrate/nitrite back to baseline levels is through dietary inorganic nitrate supplementation. My results have important implications for future investigations and also potentially offer evidence for the development of a diagnostic marker of atherosclerosis based upon nitrate. In addition my data advocates the administration of inorganic dietary nitrate in both hypertension and atherosclerosis. The use of a dietary intervention in this way represents an option that is cheap, effective and readily available.

CVD causes 1/3 of all deaths in the UK with coronary heart disease being responsible for ~50% of these deaths and 28% from stroke. Hypertension is a major risk factor for CVD and is predicted to reach a global prevalence of 30% by 2025 (Kearney *et al.*, 2005). Whilst the lipid lowering drugs, the statins, and aspirin have substantially reduced the incidence of mortality from such disease (Baigent *et al.*, 2005; Patrono *et al.*, 2005) a significant mortality remains and with increasing levels of morbidity a substantial health burden persists. There is, therefore, a need to find therapies to further reduce this health burden. Since CVD is characterised by a pathogenic state of inflammation identification of agents that might improve this state is an area of great interest and potential therapeutic utility. Previous work suggests that a dietary nitrate approach might underlie the benefits of fruit- and vegetable-rich diets, in part by suppressing inflammatory phenomena occurring at the level of the endothelium. The research carried out in this thesis largely advocates the consumption of a diet high in nitrate i.e. a natural and low cost strategy, which may be useful in protecting individuals from atherosclerotic and hypertensive disease and diseases associated with cardiovascular inflammation, thereby ultimately decreasing both healthcare costs and the number of adverse events.

Chapter 7:

Bibliography

Aamand, R, Dalsgaard, T, Jensen, FB, Simonsen, U, Roepstorff, A, Fago, A (2009) Generation of nitric oxide from nitrite by carbonic anhydrase: a possible link between metabolic activity and vasodilation. *Am J Physiol Heart Circ Physiol* **297**(6)**:** H2068-2074.

Abadeh, S, Case, PC, Harrison, R (1992) Demonstration of xanthine oxidase in human heart. *Biochem.Soc.Trans.* **20**(4)**:** 346S.

Abadeh, S, Case, PC, Harrison, R (1993) Purification of xanthine oxidase from human heart. *Biochem.Soc.Trans.* **21**(2)**:** 99S.

Adachi, T, Fukushima, T, Usami, Y, Hirano, K (1993) Binding of human xanthine oxidase to sulphated glycosaminoglycans on the endothelial-cell surface. *The Biochemical journal* **289 (Pt 2):** 523-527.

Ahluwalia, A, Foster, P, Scotland, RS, McLean, PG, Mathur, A, Perretti, M, Moncada, S, Hobbs, AJ (2004) Antiinflammatory activity of soluble guanylate cyclase: cGMP-dependent down-regulation of P-selectin expression and leukocyte recruitment. *Proc Natl Acad Sci U S A* **101**(5)**:** 1386-1391.

Ajuebor, MN, Virag, L, Flower, RJ, Perretti, M, Szabo, C (1998) Role of inducible nitric oxide synthase in the regulation of neutrophil migration in zymosan-induced inflammation. *Immunology* **95**(4)**:** 625-630.

Alp, NJ, Channon, KM (2004) Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arterioscler Thromb Vasc Biol* **24**(3)**:** 413-420.

Andrews, P, Bray, RC, Edwards, P, Shooter, KV (1964) The chemistry of xanthine oxidase. 11. Ultracentrifuge and gel-filtration studies on the milk enzyme. *Biochem J* **93**(3)**:** 627-632.

Appel, LJ, Moore, TJ, Obarzanek, E, Vollmer, WM, Svetkey, LP, Sacks, FM, Bray, GA, Vogt, TM, Cutler, JA, Windhauser, MM, Lin, PH, Karanja, N (1997) A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *N Engl J Med* **336**(16)**:** 1117-1124.

Archer, S (1993) Measurement of nitric oxide in biological models. *FASEB J* **7**(2)**:** 349-360.

Augustin, HG, Kozian, DH, Johnson, RC (1994) Differentiation of endothelial cells: analysis of the constitutive and activated endothelial cell phenotypes. *Bioessays* **16**(12)**:** 901-906.

Baigent, C, Keech, A, Kearney, PM, Blackwell, L, Buck, G, Pollicino, C, Kirby, A, Sourjina, T, Peto, R, Collins, R, Simes, R (2005) Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet* **366**(9493)**:** 1267-1278.

Ballou, DP, Zhao, Y, Brandish, PE, Marletta, MA (2002) Revisiting the kinetics of nitric oxide (NO) binding to soluble guanylate cyclase: the simple NO-binding model is incorrect. *Proc Natl Acad Sci U S A* **99**(19)**:** 12097-12101.

Basu, S, Grubina, R, Huang, J, Conradie, J, Huang, Z, Jeffers, A, Jiang, A, He, X, Azarov, I, Seibert, R, Mehta, A, Patel, R, King, SB, Hogg, N, Ghosh, A, Gladwin, MT, Kim-Shapiro, DB (2007) Catalytic generation of N2O3 by the concerted nitrite reductase and anhydrase activity of hemoglobin. *Nat Chem Biol* **3**(12)**:** 785-794.

Bath, PM, Hassall, DG, Gladwin, AM, Palmer, RM, Martin, JF (1991) Nitric oxide and prostacyclin. Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. *Arterioscler Thromb* **11**(2)**:** 254-260.

Beckman, JA, Goldfine, AB, Gordon, MB, Creager, MA (2001) Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. *Circulation* **103**(12)**:** 1618-1623.

Beckman, JS, Beckman, TW, Chen, J, Marshall, PA, Freeman, BA (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* **87**(4)**:** 1620-1624.

Beckman, JS, Koppenol, WH (1996a) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* **271**(5 Pt 1)**:** C1424-1437.

Beckman, JS, Koppenol, WH (1996b) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *American Journal of Physiology - Cell Physiology* **271**(5)**:** C1424-C1437.

Bedford, MT, Clarke, SG (2009) Protein arginine methylation in mammals: who, what, and why. *Mol Cell* **33**(1)**:** 1-13.

Beevers, G, Lip, GY, O'Brien, E (2001) ABC of hypertension: The pathophysiology of hypertension. *BMJ* **322**(7291)**:** 912-916.

Bellisarii, FI, Gallina, S, Zimarino, M, De Caterina, R (2003) Mechanisms of nitrate tolerance: potential roles of folate. *Eur J Clin Invest* **33**(11)**:** 933-940.

Benjamin, N, O'Driscoll, F, Dougall, H, Duncan, C, Smith, L, Golden, M, McKenzie, H (1994) Stomach NO synthesis. *Nature* **368**(6471)**:** 502.

Berry, CE, Hare, JM (2004) Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *J Physiol* **555**(Pt 3)**:** 589-606.

Bevilacqua, MP, Nelson, RM (1993) Endothelial-leukocyte adhesion molecules in inflammation and metastasis. *Thromb Haemost* **70**(1)**:** 152-154.

Binh, PN, Abe, Y, Tien, PG, Son le, NT, Hanh, TT, Diep do, TN, Qui le, TK, Kawano, M, Maruyama, C (2011) Plasma NOx concentrations in glucose intolerance and type 2 diabetes. A case-control study in a Vietnamese population. *J Atheroscler Thromb* **18**(4)**:** 305-311.

Bjelakovic, G, Nikolova, D, Gluud, LL, Simonetti, RG, Gluud, C (2012) Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database Syst Rev* **3:** CD007176.

Bjelakovic, G, Nikolova, D, Gluud, LL, Simonetti, RG, Gluud, C (2007) Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA* **297**(8)**:** 842-857.

Blankenberg, S, Barbaux, S, Tiret, L (2003) Adhesion molecules and atherosclerosis. *Atherosclerosis* **170**(2)**:** 191-203.

Boger, RH, Bode-Boger, SM, Thiele, W, Junker, W, Alexander, K, Frolich, JC (1997) Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. *Circulation* **95**(8)**:** 2068-2074.

Bradford, MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248-254.

Bredt, DS, Snyder, SH (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci U S A* **87**(2)**:** 682-685.

Brunner, H, Cockcroft, JR, Deanfield, J, Donald, A, Ferrannini, E, Halcox, J, Kiowski, W, Luscher, TF, Mancia, G, Natali, A, Oliver, JJ, Pessina, AC, Rizzoni, D, Rossi, GP, Salvetti, A, Spieker, LE, Taddei, S, Webb, DJ (2005) Endothelial function and dysfunction. Part II: Association with cardiovascular risk factors and diseases. A statement by the Working Group on Endothelins and Endothelial Factors of the European Society of Hypertension. *J Hypertens* **23**(2)**:** 233-246.

Bryan, NS (2006) Nitrite in nitric oxide biology: cause or consequence? A systems-based review. *Free Radic Biol Med* **41**(5)**:** 691-701.

Bryan, NS, Calvert, JW, Elrod, JW, Gundewar, S, Ji, SY, Lefer, DJ (2007) Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* **104**(48)**:** 19144-19149.

Bryan, NS, Calvert, JW, Gundewar, S, Lefer, DJ (2008) Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice. *Free Radic Biol Med* **45**(4)**:** 468-474.

Bryan, NS, Fernandez, BO, Bauer, SM, Garcia-Saura, MF, Milsom, AB, Rassaf, T, Maloney, RE, Bharti, A, Rodriguez, J, Feelisch, M (2005) Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat Chem Biol* **1**(5)**:** 290-297.

Bryan, NS, Rassaf, T, Maloney, RE, Rodriguez, CM, Saijo, F, Rodriguez, JR, Feelisch, M (2004) Cellular targets and mechanisms of nitros(yl)ation: an insight into their nature and kinetics in vivo. *Proc Natl Acad Sci U S A* **101**(12)**:** 4308-4313.

Bubb, KJ, Khambata, RS, Ahluwalia, A (2012) Sexual dimorphism in rodent models of hypertension and atherosclerosis. *Br J Pharmacol* **167**(2)**:** 298-312.

Burnier, M, Wuerzner, G, Struijker-Boudier, H, Urquhart, J (2013) Measuring, analyzing, and managing drug adherence in resistant hypertension. *Hypertension* **62**(2)**:** 218-225.

Busse, R, Edwards, G, Feletou, M, Fleming, I, Vanhoutte, PM, Weston, AH (2002) EDHF: bringing the concepts together. *Trends Pharmacol Sci* **23**(8)**:** 374-380.

Cai, H, Harrison, DG (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* **87**(10)**:** 840-844.

Cai, S, Khoo, J, Mussa, S, Alp, NJ, Channon, KM (2005) Endothelial nitric oxide synthase dysfunction in diabetic mice: importance of tetrahydrobiopterin in eNOS dimerisation. *Diabetologia* **48**(9)**:** 1933-1940.

Carlstrom, M, Persson, AE, Larsson, E, Hezel, M, Scheffer, PG, Teerlink, T, Weitzberg, E, Lundberg, JO (2011) Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension. *Cardiovasc Res* **89**(3)**:** 574-585.

Carretero, OA, Oparil, S (2000) Essential hypertension. Part I: definition and etiology. *Circulation* **101**(3)**:** 329-335.

Carswell, EA, Old, LJ, Kassel, RL, Green, S, Fiore, N, Williamson, B (1975) An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* **72**(9)**:** 3666-3670.

Cary, SP, Winger, JA, Derbyshire, ER, Marletta, MA (2006) Nitric oxide signaling: no longer simply on or off. *Trends Biochem Sci* **31**(4)**:** 231-239.

Cash, JL, White, GE, Greaves, DR (2009) Chapter 17. Zymosan-induced peritonitis as a simple experimental system for the study of inflammation. *Methods Enzymol* **461:** 379-396.

Celermajer, DS, Sorensen, KE, Bull, C, Robinson, J, Deanfield, JE (1994) Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am Coll Cardiol* **24**(6)**:** 1468-1474.

Celermajer, DS, Sorensen, KE, Gooch, VM, Spiegelhalter, DJ, Miller, OI, Sullivan, ID, Lloyd, JK, Deanfield, JE (1992) Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* **340**(8828)**:** 1111-1115.

Chan, M (2013) A global brief on hypertension: world health day 2013. *World Health Organization*.

Chan, V, Fenning, A, Levick, SP, Loch, D, Chunduri, P, Iyer, A, Teo, YL, Hoey, A, Wilson, K, Burstow, D, Brown, L (2011) Cardiovascular changes during maturation and ageing in male and female spontaneously hypertensive rats. *J Cardiovasc Pharmacol* **57**(4)**:** 469-478.

Channon, KM, Guzik, TJ (2002) Mechanisms of superoxide production in human blood vessels: relationship to endothelial dysfunction, clinical and genetic risk factors. *J Physiol Pharmacol* **53**(4 Pt 1)**:** 515-524.

Chen, G, Suzuki, H, Weston, AH (1988) Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br J Pharmacol* **95**(4)**:** 1165-1174.

Chou, TC, Yen, MH, Li, CY, Ding, YA (1998) Alterations of nitric oxide synthase expression with aging and hypertension in rats. *Hypertension* **31**(2)**:** 643-648.

Cooke, JP (2000) Does ADMA cause endothelial dysfunction? *Arterioscler Thromb Vasc Biol* **20**(9)**:** 2032-2037.

Cooke, JP, Stamler, J, Andon, N, Davies, PF, McKinley, G, Loscalzo, J (1990) Flow stimulates endothelial cells to release a nitrovasodilator that is potentiated by reduced thiol. *Am J Physiol* **259**(3 Pt 2)**:** H804-812.

Corretti, MC, Anderson, TJ, Benjamin, EJ, Celermajer, D, Charbonneau, F, Creager, MA, Deanfield, J, Drexler, H, Gerhard-Herman, M, Herrington, D, Vallance, P, Vita, J, Vogel, R (2002) Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* **39**(2)**:** 257-265.

Cosby, K, Partovi, KS, Crawford, JH, Patel, RP, Reiter, CD, Martyr, S, Yang, BK, Waclawiw, MA, Zalos, G, Xu, X, Huang, KT, Shields, H, Kim-Shapiro, DB, Schechter, AN, Cannon, RO, 3rd, Gladwin, MT (2003a) Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* **9**(12)**:** 1498-1505.

Cosby, K, Partovi, KS, Crawford, JH, Patel, RP, Reiter, CD, Martyr, S, Yang, BK, Waclawiw, MA, Zalos, G, Xu, X, Huang, KT, Shields, H, Kim-Shapiro, DB, Schechter, AN, Cannon, RO, III, Gladwin, MT (2003b) Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nature Medicine* **9**(12)**:** 1498-1505.

Crabtree, MJ, Hale, AB, Channon, KM (2011) Dihydrofolate reductase protects endothelial nitric oxide synthase from uncoupling in tetrahydrobiopterin deficiency. *Free Radic Biol Med* **50**(11)**:** 1639-1646.

d'Uscio, LV, Smith, LA, Katusic, ZS (2001) Hypercholesterolemia impairs endothelium-dependent relaxations in common carotid arteries of apolipoprotein e-deficient mice. *Stroke* **32**(11)**:** 2658-2664.

Dangel, O, Mergia, E, Karlisch, K, Groneberg, D, Koesling, D, Friebe, A (2010) Nitric oxide-sensitive guanylyl cyclase is the only nitric oxide receptor mediating platelet inhibition. *J Thromb Haemost* **8**(6)**:** 1343-1352.

Dangerfield, JP, Wang, S, Nourshargh, S (2005) Blockade of alpha6 integrin inhibits IL-1beta- but not TNF-alpha-induced neutrophil transmigration in vivo. *J Leukoc Biol* **77**(2)**:** 159-165.

Davenpeck, KL, Gauthier, TW, Lefer, AM (1994) Inhibition of endothelial-derived nitric oxide promotes P-selectin expression and actions in the rat microcirculation. *Gastroenterology* **107**(4)**:** 1050-1058.

Dawson, TM, Snyder, SH (1994) Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *J Neurosci* **14**(9)**:** 5147-5159.

De Caterina, R, Libby, P, Peng, HB, Thannickal, VJ, Rajavashisth, TB, Gimbrone, MA, Jr., Shin, WS, Liao, JK (1995) Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* **96**(1)**:** 60-68.

Dejam, A, Hunter, CJ, Pelletier, MM, Hsu, LL, Machado, RF, Shiva, S, Power, GG, Kelm, M, Gladwin, MT, Schechter, AN (2005) Erythrocytes are the major intravascular storage sites of nitrite in human blood. *Blood* **106**(2)**:** 734-739.

Dejam, A, Hunter, CJ, Tremonti, C, Pluta, RM, Hon, YY, Grimes, G, Partovi, K, Pelletier, MM, Oldfield, EH, Cannon, RO, 3rd, Schechter, AN, Gladwin, MT (2007a) Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. *Circulation* **116**(16)**:** 1821-1831.

Dejam, A, Hunter, CJ, Tremonti, C, Pluta, RM, Hon, YY, Grimes, G, Partovi, K, Pelletier, MM, Oldfield, EH, Cannon, RO, III, Schechter, AN, Gladwin, MT (2007b) Nitrite Infusion

in Humans and Nonhuman Primates. Endocrine Effects, Pharmacokinetics, and Tolerance Formation. *Circulation* **116:** 1821-1831.

Della Corte, E, Stirpe, F (1968) The regulation of rat-liver xanthine oxidase: Activation by proteolytic enzymes. *FEBS Lett* **2**(2)**:** 83-84.

Denninger, JW, Marletta, MA (1999) Guanylate cyclase and the .NO/cGMP signaling pathway. *Biochim Biophys Acta* **1411**(2-3)**:** 334-350.

Desai, A, Zhao, Y, Lankford, HA, Warren, JS (2006) Nitric oxide suppresses EPO-induced monocyte chemoattractant protein-1 in endothelial cells: implications for atherogenesis in chronic renal disease. *Lab Invest* **86**(4)**:** 369-379.

Dias, RG, Negrao, CE, Krieger, MH (2009) Nitric oxide and the cardiovascular system: cell activation, vascular reactivity and genetic variant. *Arq Bras Cardiol* **96**(1)**:** 68-75.

Dimmeler, S, Fleming, I, Fisslthaler, B, Hermann, C, Busse, R, Zeiher, AM (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**(6736)**:** 601-605.

Ding, ZM, Babensee, JE, Simon, SI, Lu, H, Perrard, JL, Bullard, DC, Dai, XY, Bromley, SK, Dustin, ML, Entman, ML, Smith, CW, Ballantyne, CM (1999) Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J Immunol* **163**(9)**:** 5029-5038.

Dixon, M, Thurlow, S (1924) Studies on Xanthine Oxidase: The Reduction of Nitrates. *The Biochemical journal* **18**(5)**:** 989-992.

Doel, JJ, Benjamin, N, Hector, MP, Rogers, M, Allaker, RP (2005) Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur J Oral Sci* **113**(1)**:** 14-19.

Drab, M, Verkade, P, Elger, M, Kasper, M, Lohn, M, Lauterbach, B, Menne, J, Lindschau, C, Mende, F, Luft, FC, Schedl, A, Haller, H, Kurzchalia, TV (2001) Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* **293**(5539)**:** 2449-2452.

Drexler, H (1997) Endothelial dysfunction: clinical implications. *Prog Cardiovasc Dis* **39**(4)**:** 287-324.

Drexler, H, Zeiher, AM (1991) Endothelial function in human coronary arteries in vivo. Focus on hypercholesterolemia. *Hypertension* **18**(4 Suppl)**:** II90-99.

Dupont, GP, Huecksteadt, TP, Marshall, BC, Ryan, US, Michael, JR, Hoidal, JR (1992) Regulation of xanthine dehydrogenase and xanthine oxidase activity and gene expression in cultured rat pulmonary endothelial cells. *The Journal of Clinical Investigation* **89**(1)**:** 197-202.

Duranski, MR, Greer, JJ, Dejam, A, Jaganmohan, S, Hogg, N, Langston, W, Patel, RP, Yet, SF, Wang, X, Kevil, CG, Gladwin, MT, Lefer, DJ (2005) Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest* **115**(5)**:** 1232-1240.

EFSA (2008) Nitrate in vegetables. Scientific opinion of the panel on contaminants in the food chain. *The EFSA Journal* **689:** 1-79.

Egan, BM, Zhao, Y, Axon, RN (2010) US trends in prevalence, awareness, treatment, and control of hypertension, 1988-2008. *JAMA* **303**(20)**:** 2043-2050.

Eger, BT, Okamoto, K, Enroth, C, Sato, M, Nishino, T, Pai, EF (2000) Purification, crystallization and preliminary X-ray diffraction studies of xanthine dehydrogenase and xanthine oxidase isolated from bovine milk. *Acta Crystallogr D Biol Crystallogr* **56**(Pt 12)**:** 1656-1658.

Enroth, C, Eger, BT, Okamoto, K, Nishino, T, Pai, EF (2000) Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: structure-based mechanism of conversion. *Proc Natl Acad Sci U S A* **97**(20)**:** 10723-10728.

Evans, DF, Pye, G, Bramley, R, Clark, AG, Dyson, TJ, Hardcastle, JD (1988) Measurement of gastrointestinal pH profiles in normal ambulant human subjects. *Gut* **29**(8)**:** 1035-1041.

Falaschetti, E, Chaudhury, M, Mindell, J, Poulter, N (2009) Continued improvement in hypertension management in England: results from the Health Survey for England 2006. *Hypertension* **53**(3)**:** 480-486.

Feron, O, Dessy, C, Desager, JP, Balligand, JL (2001) Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance. *Circulation* **103**(1)**:** 113-118.

Feron, O, Dessy, C, Moniotte, S, Desager, JP, Balligand, JL (1999) Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J Clin Invest* **103**(6)**:** 897-905.

Fleming, I, Busse, R (2003) Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol Regul Integr Comp Physiol* **284**(1)**:** R1-12.

Fleming, I, Fisslthaler, B, Dimmeler, S, Kemp, BE, Busse, R (2001) Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res* **88**(11)**:** E68-75.

Florey (1966) The endothelial cell. *Br Med J* **2**(5512)**:** 487-490.

Ford, PC, Lorkovic, IM (2002) Mechanistic aspects of the reactions of nitric oxide with transition-metal complexes. *Chem Rev* **102**(4)**:** 993-1018.

Forstermann, U, Closs, EI, Pollock, JS, Nakane, M, Schwarz, P, Gath, I, Kleinert, H (1994) Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* **23**(6 Pt 2)**:** 1121-1131.

Forstermann, U, Munzel, T (2006) Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* **113**(13)**:** 1708-1714.

Forstermann, U, Pollock, JS, Schmidt, HH, Heller, M, Murad, F (1991) Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* **88**(5)**:** 1788-1792.

Forstermann, U, Sessa, WC (2012) Nitric oxide synthases: regulation and function. *Eur Heart J* **33**(7)**:** 829-837, 837a-837d.

Forte, P, Copland, M, Smith, LM, Milne, E, Sutherland, J, Benjamin, N (1997) Basal nitric oxide synthesis in essential hypertension. *The Lancet* **349:** 837-842.

Fortuno, A, Olivan, S, Beloqui, O, San Jose, G, Moreno, MU, Diez, J, Zalba, G (2004) Association of increased phagocytic NADPH oxidase-dependent superoxide production with diminished nitric oxide generation in essential hypertension. *J Hypertens* **22**(11)**:** 2169-2175.

Freedman, JE, Loscalzo, J, Benoit, SE, Valeri, CR, Barnard, MR, Michelson, AD (1996) Decreased platelet inhibition by nitric oxide in two brothers with a history of arterial thrombosis. *J Clin Invest* **97**(4)**:** 979-987.

Frey, RS, Ushio-Fukai, M, Malik, AB (2009) NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology. *Antioxid Redox Signal* **11**(4)**:** 791-810.

Friedl, HP, Smith, DJ, Till, GO, Thomson, PD, Louis, DS, Ward, PA (1990) Ischemia-reperfusion in humans. Appearance of xanthine oxidase activity. *American Journal of Pathology* **136**(3)**:** 491-495.

Frostegard, J, Ulfgren, AK, Nyberg, P, Hedin, U, Swedenborg, J, Andersson, U, Hansson, GK (1999) Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* **145**(1)**:** 33-43.

Fukuto, JM, Cho, JY, Switzer, CH (2000) The chemical proerties of nitric oxide and related nitrogen oxides. In: *Nitric oxide: biology and pathobiology*, Ignarro, LJ (ed), pp 23-40. San Diego: Academic Press.

Fulton, D, Gratton, JP, McCabe, TJ, Fontana, J, Fujio, Y, Walsh, K, Franke, TF, Papapetropoulos, A, Sessa, WC (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**(6736)**:** 597-601.

Furchgott, RF, Bhadrakom, S (1953) Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. *J Pharmacol Exp Ther* **108**(2)**:** 129-143.

Furchgott, RF, Zawadzki, JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**(5789)**:** 373-376.

Furfine, ES, Harmon, MF, Paith, JE, Garvey, EP (1993) Selective inhibition of constitutive nitric oxide synthase by L-NG-nitroarginine. *Biochemistry* **32**(33)**:** 8512-8517.

Furfine, ES, Harmon, MF, Paith, JE, Knowles, RG, Salter, M, Kiff, RJ, Duffy, C, Hazelwood, R, Oplinger, JA, Garvey, EP (1994) Potent and selective inhibition of human nitric oxide synthases. Selective inhibition of neuronal nitric oxide synthase by S-methyl-L-thiocitrulline and S-ethyl-L-thiocitrulline. *J Biol Chem* **269**(43)**:** 26677-26683.

Gaboury, J, Woodman, RC, Granger, DN, Reinhardt, P, Kubes, P (1993) Nitric oxide prevents leukocyte adherence: role of superoxide. *Am J Physiol* **265**(3 Pt 2)**:** H862-867.

Gago, B, Lundberg, JO, Barbosa, RM, Laranjinha, J (2007) Red wine-dependent reduction of nitrite to nitric oxide in the stomach. *Free Radic Biol Med* **43**(9)**:** 1233-1242.

Ganten, D, Wagner, J, Zeh, K, Bader, M, Michel, JB, Paul, M, Zimmermann, F, Ruf, P, Hilgenfeldt, U, Ganten, U (1992) Species specificity of renin kinetics in transgenic rats harboring the human renin and angiotensinogen genes. *Proceedings of the National Academy of Sciences* **89**(16)**:** 7806-7810.

Garcia-Cardena, G, Fan, R, Shah, V, Sorrentino, R, Cirino, G, Papapetropoulos, A, Sessa, WC (1998) Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* **392**(6678)**:** 821-824.

Garcia-Cardena, G, Martasek, P, Masters, BS, Skidd, PM, Couet, J, Li, S, Lisanti, MP, Sessa, WC (1997) Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. *J Biol Chem* **272**(41)**:** 25437-25440.

Gauthier, TW, Scalia, R, Murohara, T, Guo, JP, Lefer, AM (1995) Nitric oxide protects against leukocyte-endothelium interactions in the early stages of hypercholesterolemia. *Arterioscler Thromb Vasc Biol* **15**(10)**:** 1652-1659.

Gautier, C, van Faassen, E, Mikula, I, Martasek, P, Slama-Schwok, A (2006) Endothelial nitric oxide synthase reduces nitrite anions to NO under anoxia. *Biochem Biophys Res Commun* **341**(3)**:** 816-821.

Gery, I, Waksman, BH (1972) Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). *J Exp Med* **136**(1)**:** 143-155.

Getting, SJ, Flower, RJ, Perretti, M (1997) Inhibition of neutrophil and monocyte recruitment by endogenous and exogenous lipocortin 1. *Br J Pharmacol* **120**(6)**:** 1075-1082.

Ghosh, SM, Kapil, V, Fuentes-Calvo, I, Bubb, KJ, Pearl, V, Milsom, AB, Khambata, R, Maleki-Toyserkani, S, Yousuf, M, Benjamin, N, Webb, AJ, Caulfield, MJ, Hobbs, AJ, Ahluwalia, A (2013) Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. *Hypertension* **61**(5)**:** 1091-1102.

Giaid, A, Saleh, D (1995) Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med* **333**(4)**:** 214-221.

Gladwin, MT, Shelhamer, JH, Schechter, AN, Pease-Fye, ME, Waclawiw, MA, Panza, JA, Ognibene, FP, Cannon, RO, 3rd (2000) Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. *Proc Natl Acad Sci U S A* **97**(21)**:** 11482-11487.

Godber, BL, Doel, JJ, Sapkota, GP, Blake, DR, Stevens, CR, Eisenthal, R, Harrison, R (2000) Reduction of nitrite to nitric oxide catalyzed by xanthine oxidoreductase. *J Biol Chem* **275**(11)**:** 7757-7763.

Goldblatt, H, Lynch, J, Hanzal, RF, Summerville, WW (1934) Studies on Experimental Hypertension : I. The Production of Persistent Elevation of Systolic Blood Pressure by Means of Renal Ischemia. *J Exp Med* **59**(3)**:** 347-379.

Gonzalez-Gay, MA, Szekanecz, Z, Popa, CD, Dessein, P (2012) Atherosclerosis in rheumatoid arthritis. *Mediators Inflamm* **2012:** 489608.

Govers, R, Rabelink, TJ (2001) Cellular regulation of endothelial nitric oxide synthase. *Am J Physiol Renal Physiol* **280**(2)**:** F193-206.

Govoni, M, Jansson, EA, Weitzberg, E, Lundberg, JO (2008) The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* **19**(4)**:** 333-337.

Grau, M, Hendgen-Cotta, UB, Brouzos, P, Drexhage, C, Rassaf, T, Lauer, T, Dejam, A, Kelm, M, Kleinbongard, P (2007a) Recent methodological advances in the analysis of nitrite in the human circulation: nitrite as a biochemical parameter of the L-arginine/NO pathway. *J Chromatogr B Analyt Technol Biomed Life Sci* **851**(1-2)**:** 106-123.

Grau, M, Hendgen-Cotta, UB, Brouzos, P, Drexhage, C, Rassaf, T, Lauer, T, Dejam, A, Kelm, M, Kleinbongard, P (2007b) Recent methodological advances in the analysis of nitrite in the human circulation: nitrite as a biochemical parameter of the L-arginine/NO pathway. *J Chromatogr.B Analyt.Technol.Biomed.Life Sci.* **851**(1-2)**:** 106-123.

Griffith, OW, Kilbourn, RG (1996) Nitric oxide synthase inhibitors: amino acids. *Methods Enzymol* **268:** 375-392.

Group, I-C (1995) ISIS-4: a randomised factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulphate in 58,050 patients with suspected

acute myocardial infarction. ISIS-4 (Fourth International Study of Infarct Survival) Collaborative Group. *Lancet* **345**(8951)**:** 669-685.

Gruetter, CA, Barry, BK, McNamara, DB, Gruetter, DY, Kadowitz, PJ, Ignarro, L (1979) Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosoamine. *J Cyclic Nucleotide Res* **5**(3)**:** 211-224.

Grum, CM, Ragsdale, RA, Ketai, LH, Shlafer, M (1986) Absence of xanthine oxidase or xanthine dehydrogenase in the rabbit myocardium. *Biochem.Biophys.Res Commun.* **141**(3)**:** 1104-1108.

Gulick, T, Chung, MK, Pieper, SJ, Lange, LG, Schreiner, GF (1989) Interleukin 1 and tumor necrosis factor inhibit cardiac myocyte beta-adrenergic responsiveness. *Proc Natl Acad Sci U S A* **86**(17)**:** 6753-6757.

Guzik, TJ, Korbut, R, Adamek-Guzik, T (2003) Nitric oxide and superoxide in inflammation and immune regulation. *J Physiol Pharmacol* **54**(4)**:** 469-487.

Guzik, TJ, Sadowski, J, Guzik, B, Jopek, A, Kapelak, B, Przybylowski, P, Wierzbicki, K, Korbut, R, Harrison, DG, Channon, KM (2006a) Coronary artery superoxide production and nox isoform expression in human coronary artery disease. *Arterioscler Thromb Vasc Biol* **26**(2)**:** 333-339.

Guzik, TJ, Sadowski, J, Guzik, B, Jopek, A, Kapelak, B, Przybylowski, P, Wierzbicki, K, Korbut, R, Harrison, DG, Channon, KM (2006b) Coronary Artery Superoxide Production and Nox Isoform Expression in Human Coronary Artery Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26:** 333-339.

Halcox, JP, Donald, AE, Ellins, E, Witte, DR, Shipley, MJ, Brunner, EJ, Marmot, MG, Deanfield, JE (2009) Endothelial function predicts progression of carotid intima-media thickness. *Circulation* **119**(7)**:** 1005-1012.

Halcox, JP, Narayanan, S, Cramer-Joyce, L, Mincemoyer, R, Quyyumi, AA (2001) Characterization of endothelium-derived hyperpolarizing factor in the human forearm microcirculation. *Am J Physiol Heart Circ Physiol* **280**(6)**:** H2470-2477.

Hansson, GK, Libby, P (2006) The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol* **6**(7)**:** 508-519.

Harris, CM, Massey, V (1997) The reaction of reduced xanthine dehydrogenase with molecular oxygen. Reaction kinetics and measurement of superoxide radical. *J Biol Chem* **272**(13)**:** 8370-8379.

Harrison, DG (1997) Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest* **100**(9)**:** 2153-2157.

Hasdai, D, Scheinowitz, M, Leibovitz, E, Sclarovsky, S, Eldar, M, Barak, V (1996) Increased serum concentrations of interleukin-1 beta in patients with coronary artery disease. *Heart* **76**(1)**:** 24-28.

Hazen, SL (2010) Neutrophils, hypercholesterolemia, and atherogenesis. *Circulation* **122**(18)**:** 1786-1788.

Heinrich, TA, da Silva, RS, Miranda, KM, Switzer, CH, Wink, DA, Fukuto, JM (2013) Biological nitric oxide signalling: chemistry and terminology. *Br J Pharmacol* **169**(7)**:** 1417-1429.

Hellsten-Westing, Y (1993) Immunohistochemical localization of xanthine oxidase in human cardiac and skeletal muscle. *Histochemistry* **100**(3)**:** 215-222.

Hendgen-Cotta, UB, Merx, MW, Shiva, S, Schmitz, J, Becher, S, Klare, JP, Steinhoff, HJ, Goedecke, A, Schrader, J, Gladwin, MT, Kelm, M, Rassaf, T (2008) Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* **105**(29)**:** 10256-10261.

Herskowitz, A, Choi, S, Ansari, AA, Wesselingh, S (1995) Cytokine mRNA expression in postischemic/reperfused myocardium. *Am J Pathol* **146**(2)**:** 419-428.

Hevel, JM, White, KA, Marletta, MA (1991) Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J Biol Chem* **266**(34)**:** 22789-22791.

Higashi, Y, Sasaki, S, Nakagawa, K, Matsuura, H, Oshima, T, Chayama, K (2002) Endothelial function and oxidative stress in renovascular hypertension. *N Engl J Med* **346**(25)**:** 1954-1962.

Hille, R (2013) The molybdenum oxotransferases and related enzymes. *Dalton Trans* **42**(9)**:** 3029-3042.

Hille, R, Massey, V (1981) Studies on the oxidative half-reaction of xanthine oxidase. *J Biol Chem* **256**(17)**:** 9090-9095.

Hille, R, Nishino, T (1995) Flavoprotein structure and mechanism. 4. Xanthine oxidase and xanthine dehydrogenase. *FASEB J* **9**(11)**:** 995-1003.

Hirata, K, Akita, H, Yokoyama, M (1991) Oxidized low density lipoprotein inhibits bradykinin-induced phosphoinositide hydrolysis in cultured bovine aortic endothelial cells. *FEBS Lett* **287**(1-2)**:** 181-184.

Hord, NG, Tang, Y, Bryan, NS (2009) Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *Am J Clin Nutr* **90**(1)**:** 1-10.

Houston, M, Estevez, A, Chumley, P, Aslan, M, Marklund, S, Parks, DA, Freeman, BA (1999) Binding of Xanthine Oxidase to Vascular Endothelium: KINETIC CHARACTERIZATION AND OXIDATIVE IMPAIRMENT OF NITRIC OXIDE-DEPENDENT SIGNALING. *Journal of Biological Chemistry* **274**(8)**:** 4985-4994.

Huang, L, Borniquel, S, Lundberg, JO (2010) Enhanced xanthine oxidoreductase expression and tissue nitrate reduction in germ free mice. *Nitric Oxide* **22**(2)**:** 191-195.

Huang, Z, Shiva, S, Kim-Shapiro, DB, Patel, RP, Ringwood, LA, Irby, CE, Huang, KT, Ho, C, Hogg, N, Schechter, AN, Gladwin, MT (2005) Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. *J Clin Invest* **115**(8)**:** 2099-2107.

Ignarro, LJ (2002) Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J Physiol Pharmacol* **53**(4 Pt 1)**:** 503-514.

Ignarro, LJ, Buga, GM, Wood, KS, Byrns, RE, Chaudhuri, G (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* **84**(24)**:** 9265-9269.

Ignarro, LJ, Degnan, JN, Baricos, WH, Kadowitz, PJ, Wolin, MS (1982) Activation of purified guanylate cyclase by nitric oxide requires heme. Comparison of heme-deficient, heme-reconstituted and heme-containing forms of soluble enzyme from bovine lung. *Biochim Biophys Acta* **718**(1)**:** 49-59.

Ignarro, LJ, Fukuto, JM, Griscavage, JM, Rogers, NE, Byrns, RE (1993) Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci U S A* **90**(17)**:** 8103-8107.

Jadert, C, Petersson, J, Massena, S, Ahl, D, Grapensparr, L, Holm, L, Lundberg, JO, Phillipson, M (2012) Decreased leukocyte recruitment by inorganic nitrate and nitrite in microvascular inflammation and NSAID-induced intestinal injury. *Free Radic Biol Med* **52**(3)**:** 683-692.

Jadert, C, Phillipson, M, Holm, L, Lundberg, JO, Borniquel, S (2014) Preventive and therapeutic effects of nitrite supplementation in experimental inflammatory bowel disease. *Redox Biol* **2:** 73-81.

Jansson, EA, Huang, L, Malkey, R, Govoni, M, Nihlen, C, Olsson, A, Stensdotter, M, Petersson, J, Holm, L, Weitzberg, E, Lundberg, JO (2008) A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. *Nat Chem Biol* **4**(7)**:** 411-417.

Jarasch, ED, Bruder, G, Heid, HW (1986) Significance of xanthine oxidase in capillary endothelial cells. *Acta Physiol Scand.Suppl* **548:** 39-46.

Jarasch, ED, Grund, C, Bruder, G, Heid, HW, Keenan, TW, Franke, WW (1981) Localization of xanthine oxidase in mammary-gland epithelium and capillary endothelium. *Cell* **25**(1)**:** 67-82.

Jung, O, Gechter, JL, Wunder, C, Paulke, A, Bartel, C, Geiger, H, Toennes, SW (2013) Resistant hypertension? Assessment of adherence by toxicological urine analysis. *J Hypertens* **31**(4)**:** 766-774.

Kanazawa, K, Kawashima, S, Mikami, S, Miwa, Y, Hirata, K, Suematsu, M, Hayashi, Y, Itoh, H, Yokoyama, M (1996) Endothelial constitutive nitric oxide synthase protein and mRNA increased in rabbit atherosclerotic aorta despite impaired endothelium-dependent vascular relaxation. *Am J Pathol* **148**(6)**:** 1949-1956.

Kang, D-H, Nakagawa, T, Feng, L, Watanabe, S, Han, L, Mazzali, M, Truong, L, Harris, R, Johnson, RJ (2002) A Role for Uric Acid in the Progression of Renal Disease. *Journal of the American Society of Nephrology* **13**(12)**:** 2888-2897.

Kapil, V, Haydar, SM, Pearl, V, Lundberg, JO, Weitzberg, E, Ahluwalia, A (2013) Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic Biol Med* **55:** 93-100.

Kapil, V, Milsom, AB, Okorie, M, Maleki-Toyserkani, S, Akram, F, Rehman, F, Arghandawi, S, Pearl, V, Benjamin, N, Loukogeorgakis, S, Macallister, R, Hobbs, AJ, Webb, AJ, Ahluwalia, A (2010) Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension* **56**(2)**:** 274-281.

Katsuki, S, Arnold, W, Mittal, C, Murad, F (1977) Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cyclic Nucleotide Res* **3**(1)**:** 23-35.

Kayyali, US, Donaldson, C, Huang, H, Abdelnour, R, Hassoun, PM (2001) Phosphorylation of xanthine dehydrogenase/oxidase in hypoxia. *J Biol.Chem.* **276**(17)**:** 14359-14365.

Kearney, PM, Whelton, M, Reynolds, K, Muntner, P, Whelton, PK, He, J (2005) Global burden of hypertension: analysis of worldwide data. *Lancet* **365**(9455)**:** 217-223.

Keeley, EC, Boura, JA, Grines, CL (2006) Comparison of primary and facilitated percutaneous coronary interventions for ST-elevation myocardial infarction: quantitative review of randomised trials. *Lancet* **367**(9510)**:** 579-588.

Kelley, EE, Hock, T, Khoo, NKH, Richardson, GR, Johnson, KK, Powell, PC, Giles, GI, Agarwal, A, Lancaster, Jr., Tarpey, MM (2006) Moderate hypoxia induces xanthine

oxidoreductase activity in arterial endothelial cells. *Free Radical Biology and Medicine* **40:** 952-959.

Kelm, M (1999) Nitric oxide metabolism and breakdown. *Biochim Biophys Acta* **1411**(2-3)**:** 273-289.

Kelm, M, Feelisch, M, Krebber, T, Deussen, A, Motz, W, Strauer, BE (1995) Role of nitric oxide in the regulation of coronary vascular tone in hearts from hypertensive rats. Maintenance of nitric oxide-forming capacity and increased basal production of nitric oxide. *Hypertension* **25**(2)**:** 186-193.

Kelm, M, Preik-Steinhoff, H, Preik, M, Strauer, BE (1999) Serum nitrite sensitively reflects endothelial NO formation in human forearm vasculature: evidence for biochemical assessment of the endothelial L-arginine-NO pathway. *Cardiovasc Res* **41**(3)**:** 765-772.

Kelm, M, Schafer, S, Dahmann, R, Dolu, B, Perings, S, Decking, UK, Schrader, J, Strauer, BE (1997) Nitric oxide induced contractile dysfunction is related to a reduction in myocardial energy generation. *Cardiovasc Res* **36**(2)**:** 185-194.

Kevil, CG, Kolluru, GK, Pattillo, CB, Giordano, T (2011) Inorganic nitrite therapy: historical perspective and future directions. *Free Radic Biol Med* **51**(3)**:** 576-593.

Kharitonov, VG, Sharma, VS, Magde, D, Koesling, D (1997) Kinetics of nitric oxide dissociation from five- and six-coordinate nitrosyl hemes and heme proteins, including soluble guanylate cyclase. *Biochemistry* **36**(22)**:** 6814-6818.

Khosla, UM, Zharikov, S, Finch, JL, Nakagawa, T, Roncal, C, Mu, W, Krotova, K, Block, ER, Prabhakar, S, Johnson, RJ (2005) Hyperuricemia induces endothelial dysfunction. *Kidney Int* **67**(5)**:** 1739-1742.

Kinlay, S, Behrendt, D, Wainstein, M, Beltrame, J, Fang, JC, Creager, MA, Selwyn, AP, Ganz, P (2001) Role of endothelin-1 in the active constriction of human atherosclerotic coronary arteries. *Circulation* **104**(10)**:** 1114-1118.

Klatt, P, Schmidt, K, Brunner, F, Mayer, B (1994) Inhibitors of brain nitric oxide synthase. Binding kinetics, metabolism, and enzyme inactivation. *J Biol Chem* **269**(3)**:** 1674-1680.

Kleinbongard, P, Dejam, A, Lauer, T, Rassaf, T, Schindler, A, Picker, O, Scheeren, T, Godecke, A, Schrader, J, Schulz, R, Heusch, G, Schaub, GA, Bryan, NS, Feelisch, M, Kelm, M (2003) Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic Biol Med* **35**(7)**:** 790-796.

Knowles, RG, Moncada, S (1994) Nitric oxide synthases in mammals. *Biochem J* **298 (Pt 2):** 249-258.

Kojda, G, Harrison, D (1999) Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovascular Research* **43**(3)**:** 652-671.

Kubes, P, Granger, DN (1996) Leukocyte-endothelial cell interactions evoked by mast cells. *Cardiovasc Res* **32**(4)**:** 699-708.

Kubes, P, Granger, DN (1992) Nitric oxide modulates microvascular permeability. *Am J Physiol* **262**(2 Pt 2)**:** H611-615.

Kubes, P, Suzuki, M, Granger, DN (1991) Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* **88**(11)**:** 4651-4655.

Kurose, I, Kubes, P, Wolf, R, Anderson, DC, Paulson, J, Miyasaka, M, Granger, DN (1993) Inhibition of nitric oxide production. Mechanisms of vascular albumin leakage. *Circ Res* **73**(1)**:** 164-171.

Laakso, J, Mervaala, E, Himberg, J-J, Teräväinen, T-L, Karppanen, H, Vapaatalo, H, Lapatto, R (1998a) Increased Kidney Xanthine Oxidoreductase Activity in Salt-Induced Experimental Hypertension. *Hypertension* **32**(5)**:** 902-906.

Laakso, J, Mervaala, E, Himberg, JJ, Teravainen, TL, Karppanen, H, Vapaatalo, H, Lapatto, R (1998b) Increased kidney xanthine oxidoreductase activity in salt-induced experimental hypertension. *Hypertension* **32**(5)**:** 902-906.

Laakso, JT, Teravainen, TL, Martelin, E, Vaskonen, T, Lapatto, R (2004) Renal xanthine oxidoreductase activity during development of hypertension in spontaneously hypertensive rats. *J Hypertens* **22**(7)**:** 1333-1340.

Lancaster, JR, Jr. (1994) Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc Natl Acad Sci U S A* **91**(17)**:** 8137-8141.

Landmesser, U, Spiekermann, S, Preuss, C, Sorrentino, S, Fischer, D, Manes, C, Mueller, M, Drexler, H (2007) Angiotensin II induces endothelial xanthine oxidase activation: role for endothelial dysfunction in patients with coronary disease. *Arterioscler.Thromb.Vasc.Biol.* **27**(4)**:** 943-948.

Langlois, PF, Gawryl, MS (1988) Detection of the terminal complement complex in patient plasma following acute myocardial infarction. *Atherosclerosis* **70**(1-2)**:** 95-105.

Lanzillo, JJ, Yu, FS, Stevens, J, Hassoun, PM (1996) Determination of xanthine dehydrogenase mRNA by a reverse transcription-coupled competitive quantitative polymerase chain reaction assay: regulation in rat endothelial cells by hypoxia and hyperoxia. *Arch.Biochem.Biophys.* **335**(2)**:** 377-380.

Larsen, FJ, Ekblom, B, Sahlin, K, Lundberg, JO, Weitzberg, E (2006) Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med* **355**(26)**:** 2792-2793.

Latini, R, Bianchi, M, Correale, E, Dinarello, CA, Fantuzzi, G, Fresco, C, Maggioni, AP, Mengozzi, M, Romano, S, Shapiro, L, et al. (1994) Cytokines in acute myocardial infarction: selective increase in circulating tumor necrosis factor, its soluble receptor, and interleukin-1 receptor antagonist. *J Cardiovasc Pharmacol* **23**(1)**:** 1-6.

Lauer, T, Preik, M, Rassaf, T, Strauer, BE, Deussen, A, Feelisch, M, Kelm, M (2001) Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proc Natl Acad Sci U S A* **98**(22)**:** 12814-12819.

Laurent, S, Schlaich, M, Esler, M (2012) New drugs, procedures, and devices for hypertension. *Lancet* **380**(9841)**:** 591-600.

Laursen, JB, Somers, M, Kurz, S, McCann, L, Warnholtz, A, Freeman, BA, Tarpey, M, Fukai, T, Harrison, DG (2001) Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* **103**(9)**:** 1282-1288.

Law, MR, Morris, JK, Wald, NJ (2009) Use of blood pressure lowering drugs in the prevention of cardiovascular disease: meta-analysis of 147 randomised trials in the context of expectations from prospective epidemiological studies. *BMJ* **338:** b1665.

Lee, VM, Quinn, PA, Jennings, SC, Ng, LL (2003) Neutrophil activation and production of reactive oxygen species in pre-eclampsia. *J Hypertens* **21**(2)**:** 395-402.

Lefer, AM, Murohara, T, Buerke, M (1994) Effects of taprostene on neutrophil-endothelial interactions in isolated coronary arteries. *Methods Find Exp Clin Pharmacol* **16**(9)**:** 623-631.

Lefer, DJ, Jones, SP, Girod, WG, Baines, A, Grisham, MB, Cockrell, AS, Huang, PL, Scalia, R (1999) Leukocyte-endothelial cell interactions in nitric oxide synthase-deficient mice. *Am J Physiol* **276**(6 Pt 2)**:** H1943-1950.

Levine, GN, Frei, B, Koulouris, SN, Gerhard, MD, Keaney, JF, Jr., Vita, JA (1996) Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation* **93**(6)**:** 1107-1113.

Lewis, RS, Deen, WM (1994) Kinetics of the reaction of nitric oxide with oxygen in aqueous solutions. *Chem Res Toxicol* **7**(4)**:** 568-574.

Ley, K, Laudanna, C, Cybulsky, MI, Nourshargh, S (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* **7**(9)**:** 678-689.

Li, H, Cui, H, Kundu, TK, Alzawahra, W, Zweier, JL (2008) Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. *J Biol Chem* **283**(26)**:** 17855-17863.

Li, H, Duncan, C, Townend, J, Killham, K, Smith, LM, Johnston, P, Dykhuizen, R, Kelly, D, Golden, M, Benjamin, N, Leifert, C (1997) Nitrate-reducing bacteria on rat tongues. *Appl Environ Microbiol* **63**(3)**:** 924-930.

Li, H, Kundu, TK, Zweier, JL (2009) Characterization of the magnitude and mechanism of aldehyde oxidase-mediated nitric oxide production from nitrite. *J Biol Chem* **284**(49)**:** 33850-33858.

Li, H, Samouilov, A, Liu, X, Zweier, JL (2004a) Characterization of the effects of oxygen on xanthine oxidase-mediated nitric oxide formation. *J Biol.Chem.* **279**(17)**:** 16939-16946.

Li, H, Samouilov, A, Liu, X, Zweier, JL (2003) Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrate reduction: evaluation of its role in nitrite and nitric oxide generation in anoxic tissues. *Biochemistry* **42**(4)**:** 1150-1159.

Li, H, Samouilov, A, Liu, X, Zweier, JL (2001a) Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. *J Biol.Chem.* **276**(27)**:** 24482-24489.

Li, H, Samouilov, A, Liu, X, Zweier, JL (2001b) Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. *J Biol Chem* **276**(27)**:** 24482-24489.

Li, YJ, Ding, WH, Gao, W, Huo, Y, Hong, T, Zhu, RY, Ma, DL (2004b) [The protective effect of interleukin-1 receptor antagonist on postischemic reperfused myocardium and its possible mechanism]. *Zhonghua Yi Xue Za Zhi* **84**(7)**:** 548-553.

Lim, SS, Vos, T, Flaxman, AD, Danaei, G, Shibuya, K, Adair-Rohani, H, Amann, M, Anderson, HR, Andrews, KG, Aryee, M, Atkinson, C, Bacchus, LJ, Bahalim, AN, Balakrishnan, K, Balmes, J, Barker-Collo, S, Baxter, A, Bell, ML, Blore, JD, Blyth, F, Bonner, C, Borges, G, Bourne, R, Boussinesq, M, Brauer, M, Brooks, P, Bruce, NG, Brunekreef, B, Bryan-Hancock, C, Bucello, C, Buchbinder, R, Bull, F, Burnett, RT, Byers, TE, Calabria, B, Carapetis, J, Carnahan, E, Chafe, Z, Charlson, F, Chen, H, Chen, JS, Cheng, AT, Child, JC, Cohen, A, Colson, KE, Cowie, BC, Darby, S, Darling, S, Davis, A, Degenhardt, L, Dentener, F, Des Jarlais, DC, Devries, K, Dherani, M, Ding, EL, Dorsey, ER, Driscoll, T, Edmond, K, Ali, SE, Engell, RE, Erwin, PJ, Fahimi, S, Falder, G, Farzadfar, F, Ferrari, A, Finucane, MM, Flaxman, S, Fowkes, FG, Freedman, G, Freeman, MK, Gakidou, E, Ghosh, S, Giovannucci, E, Gmel, G, Graham, K, Grainger, R, Grant, B,

Gunnell, D, Gutierrez, HR, Hall, W, Hoek, HW, Hogan, A, Hosgood, HD, 3rd, Hoy, D, Hu, H, Hubbell, BJ, Hutchings, SJ, Ibeanusi, SE, Jacklyn, GL, Jasrasaria, R, Jonas, JB, Kan, H, Kanis, JA, Kassebaum, N, Kawakami, N, Khang, YH, Khatibzadeh, S, Khoo, JP, Kok, C, Laden, F, Lalloo, R, Lan, Q, Lathlean, T, Leasher, JL, Leigh, J, Li, Y, Lin, JK, Lipshultz, SE, London, S, Lozano, R, Lu, Y, Mak, J, Malekzadeh, R, Mallinger, L, Marcenes, W, March, L, Marks, R, Martin, R, McGale, P, McGrath, J, Mehta, S, Mensah, GA, Merriman, TR, Micha, R, Michaud, C, Mishra, V, Mohd Hanafiah, K, Mokdad, AA, Morawska, L, Mozaffarian, D, Murphy, T, Naghavi, M, Neal, B, Nelson, PK, Nolla, JM, Norman, R, Olives, C, Omer, SB, Orchard, J, Osborne, R, Ostro, B, Page, A, Pandey, KD, Parry, CD, Passmore, E, Patra, J, Pearce, N, Pelizzari, PM, Petzold, M, Phillips, MR, Pope, D, Pope, CA, 3rd, Powles, J, Rao, M, Razavi, H, Rehfuess, EA, Rehm, JT, Ritz, B, Rivara, FP, Roberts, T, Robinson, C, Rodriguez-Portales, JA, Romieu, I, Room, R, Rosenfeld, LC, Roy, A, Rushton, L, Salomon, JA, Sampson, U, Sanchez-Riera, L, Sanman, E, Sapkota, A, Seedat, S, Shi, P, Shield, K, Shivakoti, R, Singh, GM, Sleet, DA, Smith, E, Smith, KR, Stapelberg, NJ, Steenland, K, Stockl, H, Stovner, LJ, Straif, K, Straney, L, Thurston, GD, Tran, JH, Van Dingenen, R, van Donkelaar, A, Veerman, JL, Vijayakumar, L, Weintraub, R, Weissman, MM, White, RA, Whiteford, H, Wiersma, ST, Wilkinson, JD, Williams, HC, Williams, W, Wilson, N, Woolf, AD, Yip, P, Zielinski, JM, Lopez, AD, Murray, CJ, Ezzati, M, AlMazroa, MA, Memish, ZA (2010) A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **380**(9859)**:** 2224-2260.

Linder, N, Rapola, J, Raivio, KO (1999) Cellular expression of xanthine oxidoreductase protein in normal human tissues. *LAB INVEST* **79**(8)**:** 967-974.

Liu, J, Garcia-Cardena, G, Sessa, WC (1995) Biosynthesis and palmitoylation of endothelial nitric oxide synthase: mutagenesis of palmitoylation sites, cysteines-15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. *Biochemistry* **34**(38)**:** 12333-12340.

Loffredo, L, Marcoccia, A, Pignatelli, P, Andreozzi, P, Borgia, MC, Cangemi, R, Chiarotti, F, Violi, F (2007) Oxidative-stress-mediated arterial dysfunction in patients with peripheral arterial disease. *Eur Heart J* **28**(5)**:** 608-612.

Luchsinger, BP, Rich, EN, Yan, Y, Williams, EM, Stamler, JS, Singel, DJ (2005) Assessments of the chemistry and vasodilatory activity of nitrite with hemoglobin under physiologically relevant conditions. *J Inorg Biochem* **99**(4)**:** 912-921.

Ludmer, PL, Selwyn, AP, Shook, TL, Wayne, RR, Mudge, GH, Alexander, RW, Ganz, P (1986) Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med* **315**(17)**:** 1046-1051.

Lundberg, JO (2009) Cardiovascular prevention by dietary nitrate and nitrite. *Am J Physiol Heart Circ Physiol* **296**(5)**:** H1221-1223.

Lundberg, JO, Feelisch, M, Bjorne, H, Jansson, EA, Weitzberg, E (2006) Cardioprotective effects of vegetables: is nitrate the answer? *Nitric Oxide* **15**(4)**:** 359-362.

Lundberg, JO, Gladwin, MT, Ahluwalia, A, Benjamin, N, Bryan, NS, Butler, A, Cabrales, P, Fago, A, Feelisch, M, Ford, PC, Freeman, BA, Frenneaux, M, Friedman, J, Kelm, M, Kevil, CG, Kim-Shapiro, DB, Kozlov, AV, Lancaster, JR, Jr., Lefer, DJ, McColl, K, McCurry, K, Patel, RP, Petersson, J, Rassaf, T, Reutov, VP, Richter-Addo, GB, Schechter, A, Shiva, S, Tsuchiya, K, van Faassen, EE, Webb, AJ, Zuckerbraun, BS, Zweier, JL, Weitzberg, E (2009) Nitrate and nitrite in biology, nutrition and therapeutics. *Nat Chem Biol* **5**(12)**:** 865-869.

Lundberg, JO, Govoni, M (2004) Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med* **37**(3)**:** 395-400.

Lundberg, JO, Weitzberg, E, Gladwin, MT (2008) The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* **7**(2)**:** 156-167.

Lundberg, JO, Weitzberg, E, Lundberg, JM, Alving, K (1994) Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* **35**(11)**:** 1543-1546.

Lusis, AJ (2000) Atherosclerosis. *Nature* **407**(6801)**:** 233-241.

Maejima, K, Nakano, S, Himeno, M, Tsuda, S, Makiishi, H, Ito, T, Nakagawa, A, Kigoshi, T, Ishibashi, T, Nishio, M, Uchida, K (2001) Increased basal levels of plasma nitric oxide in Type 2 diabetic subjects. Relationship to microvascular complications. *J Diabetes Complications* **15**(3)**:** 135-143.

Maher, AR, Milsom, AB, Gunaruwan, P, Abozguia, K, Ahmed, I, Weaver, RA, Thomas, P, Ashrafian, H, Born, GV, James, PE, Frenneaux, MP (2008) Hypoxic modulation of exogenous nitrite-induced vasodilation in humans. *Circulation* **117**(5)**:** 670-677.

Manea, A (2010) NADPH oxidase-derived reactive oxygen species: involvement in vascular physiology and pathology. *Cell Tissue Res* **342**(3)**:** 325-339.

Marczin, N, Antonov, A, Papapetropoulos, A, Munn, DH, Virmani, R, Kolodgie, FD, Gerrity, R, Catravas, JD (1996) Monocyte-induced downregulation of nitric oxide synthase in cultured aortic endothelial cells. *Arterioscler Thromb Vasc Biol* **16**(9)**:** 1095-1103.

Marjanovic, JA, Li, Z, Stojanovic, A, Du, X (2005) Stimulatory roles of nitric-oxide synthase 3 and guanylyl cyclase in platelet activation. *J Biol Chem* **280**(45)**:** 37430-37438.

Marletta, MA (1994) Nitric oxide synthase: aspects concerning structure and catalysis. *Cell* **78**(6)**:** 927-930.

Martin, HM, Moore, KP, Bosmans, E, Davies, S, Burroughs, AK, Dhillon, AP, Tosh, D, Harrison, R (2004) Xanthine oxidoreductase is present in bile ducts of normal and cirrhotic liver. *Free Radic Biol Med* **37**(8)**:** 1214-1223.

Massey, V, Brumby, PE, Komai, H (1969) Studies on milk xanthine oxidase. Some spectral and kinetic properties. *J Biol Chem* **244**(7)**:** 1682-1691.

May, JM, Qu, ZC, Xia, L, Cobb, CE (2000) Nitrite uptake and metabolism and oxidant stress in human erythrocytes. *Am J Physiol Cell Physiol* **279**(6)**:** C1946-1954.

Mayer, B, Beretta, M (2008) The enigma of nitroglycerin bioactivation and nitrate tolerance: news, views and troubles. *Br J Pharmacol* **155**(2)**:** 170-184.

Mazzali, M, Hughes, J, Kim, Y-G, Jefferson, JA, Kang, D-H, Gordon, KL, Lan, HY, Kivlighn, S, Johnson, RJ (2001) Elevated Uric Acid Increases Blood Pressure in the Rat by a Novel Crystal-Independent Mechanism. *Hypertension* **38**(5)**:** 1101-1106.

Mazzali, M, Kanbay, M, Segal, MS, Shafiu, M, Jalal, D, Feig, DI, Johnson, RJ (2010) Uric acid and hypertension: cause or effect? *Current rheumatology reports* **12**(2)**:** 108-117.

McDonald, KK, Rouhani, R, Handlogten, ME, Block, ER, Griffith, OW, Allison, RD, Kilberg, MS (1997) Inhibition of endothelial cell amino acid transport System y+ by arginine analogs that inhibit nitric oxide synthase. *Biochim Biophys Acta* **1324**(1)**:** 133-141.

Mehta, JL, Lopez, LM, Chen, L, Cox, OE (1994) Alterations in nitric oxide synthase activity, superoxide anion generation, and platelet aggregation in systemic hypertension, and effects of celiprolol. *Am J Cardiol* **74**(9)**:** 901-905.

Mendis, S, Puska, P, Norrving, B (2011) Global atlas on cardiovascular disease prevention and control *World health Organization*.

Mensinga, TT, Speijers, GJ, Meulenbelt, J (2003) Health implications of exposure to environmental nitrogenous compounds. *Toxicol Rev* **22**(1)**:** 41-51.

Meyrelles, SS, Peotta, VA, Pereira, TM, Vasquez, EC (2011) Endothelial dysfunction in the apolipoprotein E-deficient mouse: insights into the influence of diet, gender and aging. *Lipids Health Dis* **10:** 211.

Michel, T, Li, GK, Busconi, L (1993) Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* **90**(13)**:** 6252-6256.

Michelson, AD, Benoit, SE, Furman, MI, Breckwoldt, WL, Rohrer, MJ, Barnard, MR, Loscalzo, J (1996) Effects of nitric oxide/EDRF on platelet surface glycoproteins. *Am J Physiol* **270**(5 Pt 2)**:** H1640-1648.

Millar, TM, Stevens, CR, Benjamin, N, Eisenthal, R, Harrison, R, Blake, DR (1998a) Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Lett* **427**(2)**:** 225-228.

Millar, TM, Stevens, CR, Benjamin, N, Eisenthal, R, Harrison, R, Blake, DR (1998b) Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Lett.* **427**(2)**:** 225-228.

Miyazaki, H, Matsuoka, H, Cooke, JP, Usui, M, Ueda, S, Okuda, S, Imaizumi, T (1999) Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. *Circulation* **99**(9)**:** 1141-1146.

Mizuno, M, Ito, Y, Hepburn, N, Mizuno, T, Noda, Y, Yuzawa, Y, Harris, CL, Morgan, BP, Matsuo, S (2009) Zymosan, but not lipopolysaccharide, triggers severe and progressive peritoneal injury accompanied by complement activation in a rat peritonitis model. *J Immunol* **183**(2)**:** 1403-1412.

Mocsai, A (2013) Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *J Exp Med* **210**(7)**:** 1283-1299.

Modin, A, Bjorne, H, Herulf, M, Alving, K, Weitzberg, E, Lundberg, JO (2001) Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand* **171**(1)**:** 9-16.

Moncada, S, Higgs, A (1993) The L-arginine-nitric oxide pathway. *N Engl J Med* **329**(27)**:** 2002-2012.

Moncada, S, Higgs, EA, Vane, JR (1977) Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation. *Lancet* **1**(8001)**:** 18-20.

Moncada, S, Palmer, RM, Higgs, EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43**(2)**:** 109-142.

Moore, PK, al-Swayeh, OA, Chong, NW, Evans, RA, Gibson, A (1990) L-NG-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation in vitro. *Br J Pharmacol* **99**(2)**:** 408-412.

Moore, WM, Webber, RK, Fok, KF, Jerome, GM, Kornmeier, CM, Tjoeng, FS, Currie, MG (1996) Inhibitors of human nitric oxide synthase isoforms with the carbamidine moiety as a common structural element. *Bioorg Med Chem* **4**(9)**:** 1559-1564.

Morgan, H, Stewart, CP, Hopkins, FG (1922) On the anaerobic and aerobic oxidation of xanthin and hypoxanthin by tissues and by milk. *Proceedings of The Royal Society London* **94:** 109-131.

Moshage, H, Kok, B, Huizenga, JR, Jansen, PL (1995a) Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin Chem* **41**(6 Pt 1)**:** 892-896.

Moshage, H, Kok, B, Huizenga, JR, Jansen, PL (1995b) Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin.Chem.* **41**(6 Pt 1)**:** 892-896.

Mugge, A, Elwell, JH, Peterson, TE, Hofmeyer, TG, Heistad, DD, Harrison, DG (1991) Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ Res* **69**(5)**:** 1293-1300.

Munzel, T, Daiber, A, Mulsch, A (2005a) Explaining the phenomenon of nitrate tolerance. *Circ Res* **97**(7)**:** 618-628.

Munzel, T, Daiber, A, Ullrich, V, Mulsch, A (2005b) Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. *Arterioscler Thromb Vasc Biol* **25**(8)**:** 1551-1557.

Munzel, T, Feil, R, Mulsch, A, Lohmann, SM, Hofmann, F, Walter, U (2003) Physiology and pathophysiology of vascular signaling controlled by guanosine 3',5'-cyclic monophosphate-dependent protein kinase [corrected]. *Circulation* **108**(18)**:** 2172-2183.

Murray, CJ (2013) The state of US health, 1990-2010: burden of diseases, injuries, and risk factors. *JAMA* **310**(6)**:** 591-608.

Murray, CJ, Richards, MA, Newton, JN, Fenton, KA, Anderson, HR, Atkinson, C, Bennett, D, Bernabe, E, Blencowe, H, Bourne, R, Braithwaite, T, Brayne, C, Bruce, NG, Brugha, TS, Burney, P, Dherani, M, Dolk, H, Edmond, K, Ezzati, M, Flaxman, AD, Fleming, TD, Freedman, G, Gunnell, D, Hay, RJ, Hutchings, SJ, Ohno, SL, Lozano, R, Lyons, RA,

Marcenes, W, Naghavi, M, Newton, CR, Pearce, N, Pope, D, Rushton, L, Salomon, JA, Shibuya, K, Vos, T, Wang, H, Williams, HC, Woolf, AD, Lopez, AD, Davis, A (2010) UK health performance: findings of the Global Burden of Disease Study 2010. *Lancet* **381**(9871)**:** 997-1020.

Mustard, JF, Kinlough-Rathbone, RL, Packham, MA (1980) Prostaglandins and platelets. *Annu Rev Med* **31:** 89-96.

Naderi, SH, Bestwick, JP, Wald, DS (2012) Adherence to drugs that prevent cardiovascular disease: meta-analysis on 376,162 patients. *Am J Med* **125**(9)**:** 882-887 e881.

Nakashima, Y, Plump, AS, Raines, EW, Breslow, JL, Ross, R (1994) ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb* **14**(1)**:** 133-140.

Naruko, T, Furukawa, A, Yunoki, K, Komatsu, R, Nakagawa, M, Matsumura, Y, Shirai, N, Sugioka, K, Takagi, M, Hozumi, T, Itoh, A, Haze, K, Yoshiyama, M, Becker, AE, Ueda, M (2010) Increased expression and plasma levels of myeloperoxidase are closely related to the presence of angiographically-detected complex lesion morphology in unstable angina. *Heart* **96**(21)**:** 1716-1722.

Naruko, T, Ueda, M, Haze, K, van der Wal, AC, van der Loos, CM, Itoh, A, Komatsu, R, Ikura, Y, Ogami, M, Shimada, Y, Ehara, S, Yoshiyama, M, Takeuchi, K, Yoshikawa, J, Becker, AE (2002) Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* **106**(23)**:** 2894-2900.

Nichols, M, Townsend, N, Scarborough, P, Rayner, M (2012) European cardiovascular disease statistics 2012 edition. *European cardiovascular disease statistics*.

Nielsen, HV, Christensen, JP, Andersson, EC, Marker, O, Thomsen, AR (1994a) Expression of type 3 complement receptor on activated CD8+ T cells facilitates homing to inflammatory sites. *J Immunol* **153**(5)**:** 2021-2028.

Nielsen, VG, Weinbroum, A, Tan, S, Samuelson, PN, Gelman, S, Parks, DA (1994b) Xanthine oxidoreductase release after descending thoracic aorta occlusion and reperfusion in rabbits. *J Thorac.Cardiovasc.Surg.* **107**(5)**:** 1222-1227.

Oemar, BS, Tschudi, MR, Godoy, N, Brovkovich, V, Malinski, T, Luscher, TF (1998) Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. *Circulation* **97**(25)**:** 2494-2498.

Ohara, Y, Peterson, TE, Harrison, DG (1993) Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest* **91**(6)**:** 2546-2551.

Okamoto, K, Aoki, K (1963) Development of a strain of spontaneously hypertensive rats. *Jpn Circ J* **27:** 282-293.

Okamoto, K, Eger, BT, Nishino, T, Kondo, S, Pai, EF, Nishino, T (2003) An Extremely Potent Inhibitor of Xanthine Oxidoreductase: CRYSTAL STRUCTURE OF THE ENZYME-INHIBITOR COMPLEX AND MECHANISM OF INHIBITION. *Journal of Biological Chemistry* **278**(3)**:** 1848-1855.

Okamoto, K, Kusano, T, Nishino, T (2013) Chemical nature and reaction mechanisms of the molybdenum cofactor of xanthine oxidoreductase. *Curr Pharm Des* **19**(14)**:** 2606-2614.

Ong, SLH, Vickers, JJ, Zhang, Y, McKenzie, KUS, Walsh, CE, Whitworth, JA (2007) ROLE OF XANTHINE OXIDASE IN DEXAMETHASONE-INDUCED HYPERTENSION IN RATS. *Clinical and Experimental Pharmacology and Physiology* **34**(5-6)**:** 517-519.

Oparil, S, Zaman, MA, Calhoun, DA (2003) Pathogenesis of hypertension. *Ann Intern Med* **139**(9)**:** 761-776.

Pacher, P, Nivorozhkin, A, Szabo, C (2006) Therapeutic Effects of Xanthine Oxidase Inhibitors: Renaissance Half a Century after the Discovery of Allopurinol. *Pharmacological Reviews* **58:** 87-114.

Palmer, RM, Ashton, DS, Moncada, S (1988a) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333**(6174)**:** 664-666.

Palmer, RM, Ferrige, AG, Moncada, S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**(6122)**:** 524-526.

Palmer, RM, Rees, DD, Ashton, DS, Moncada, S (1988b) L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem Biophys Res Commun* **153**(3)**:** 1251-1256.

Pannala, AS, Mani, AR, Spencer, JP, Skinner, V, Bruckdorfer, KR, Moore, KP, Rice-Evans, CA (2003a) The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. *Free Radic Biol Med* **34**(5)**:** 576-584.

Pannala, AS, Mani, AR, Spencer, JP, Skinner, V, Bruckdorfer, KR, Moore, KP, Rice-Evans, CA (2003b) The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. *Free Radic.Biol.Med.* **34**(5)**:** 576-584.

Panza, JA, Quyyumi, AA, Brush, JE, Jr., Epstein, SE (1990) Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med* **323**(1)**:** 22-27.

Papapetropoulos, A, Garcia-Cardena, G, Madri, JA, Sessa, WC (1997) Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* **100**(12)**:** 3131-3139.

Park, JB, Charbonneau, F, Schiffrin, EL (2001) Correlation of endothelial function in large and small arteries in human essential hypertension. *J Hypertens* **19**(3)**:** 415-420.

Patrono, C, Garcia Rodriguez, LA, Landolfi, R, Baigent, C (2005) Low-dose aspirin for the prevention of atherothrombosis. *N Engl J Med* **353**(22)**:** 2373-2383.

Pedersen, ED, Waje-Andreassen, U, Vedeler, CA, Aamodt, G, Mollnes, TE (2004) Systemic complement activation following human acute ischaemic stroke. *Clin Exp Immunol* **137**(1)**:** 117-122.

Pekdemir, H, Cicek, D, Camsari, A, Akkus, MN, Cin, VG, Doven, O, Parmaksiz, HT, Katircibasi, MT, Ozcan, IT (2004) The relationship between plasma endothelin-1, nitric oxide levels, and heart rate variability in patients with coronary slow flow. *Ann Noninvasive Electrocardiol* **9**(1)**:** 24-33.

Pelletier, MM, Kleinbongard, P, Ringwood, L, Hito, R, Hunter, CJ, Schechter, AN, Gladwin, MT, Dejam, A (2006a) The measurement of blood and plasma nitrite by chemiluminescence: Pitfalls and solutions. *Free Radical Biology and Medicine* **41:** 541-548.

Pelletier, MM, Kleinbongard, P, Ringwood, L, Hito, R, Hunter, CJ, Schechter, AN, Gladwin, MT, Dejam, A (2006b) The measurement of blood and plasma nitrite by chemiluminescence: pitfalls and solutions. *Free Radic Biol Med* **41**(4)**:** 541-548.

Peri, L, Pietraforte, D, Scorza, G, Napolitano, A, Fogliano, V, Minetti, M (2005) Apples increase nitric oxide production by human saliva at the acidic pH of the stomach: a

new biological function for polyphenols with a catechol group? *Free Radic Biol Med* **39**(5)**:** 668-681.

Perretti, M, Flower, RJ (1993) Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. *J Immunol* **150**(3)**:** 992-999.

Persell, SD (2011) Prevalence of resistant hypertension in the United States, 2003-2008. *Hypertension* **57**(6)**:** 1076-1080.

Petersson, J, Carlstrom, M, Schreiber, O, Phillipson, M, Christoffersson, G, Jagare, A, Roos, S, Jansson, EA, Persson, AE, Lundberg, JO, Holm, L (2009a) Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. *Free Radic Biol Med* **46**(8)**:** 1068-1075.

Petersson, J, Carlstrom, M, Schreiber, O, Phillipson, M, Christoffersson, G, Jagare, A, Roos, S, Jansson, EA, Persson, AE, Lundberg, JO, Holm, L (2009b) Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. *Free Radic.Biol Med* **46**(8)**:** 1068-1075.

Piedrahita, JA, Zhang, SH, Hagaman, JR, Oliver, PM, Maeda, N (1992) Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc Natl Acad Sci U S A* **89**(10)**:** 4471-4475.

Pinto, YM, Paul, M, Ganten, D (1998) Lessons from rat models of hypertension: from Goldblatt to genetic engineering. *Cardiovasc Res* **39**(1)**:** 77-88.

Plump, AS, Smith, JD, Hayek, T, Aalto-Setala, K, Walsh, A, Verstuyft, JG, Rubin, EM, Breslow, JL (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* **71**(2)**:** 343-353.

Pluta, RM, Oldfield, EH, Bakhtian, KD, Fathi, AR, Smith, RK, DeVroom, HL, Nahavandi, M, Woo, S, Figg, WD, Lonser, RR (2011) Safety and Feasibility of Long-term Intravenous Sodium Nitrite Infusion in Healthy Volunteers. *PLoS ONE* **6**(1)**:** e14504.

Poderoso, JJ, Peralta, JG, Lisdero, CL, Carreras, MC, Radisic, M, Schopfer, F, Cadenas, E, Boveris, A (1998) Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. *Am J Physiol* **274**(1 Pt 1)**:** C112-119.

Pollock, JS, Forstermann, U, Mitchell, JA, Warner, TD, Schmidt, HH, Nakane, M, Murad, F (1991) Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* **88**(23)**:** 10480-10484.

Poss, WB, Huecksteadt, TP, Panus, PC, Freeman, BA, Hoidal, JR (1996) Regulation of xanthine dehydrogenase and xanthine oxidase activity by hypoxia. *Am J Physiol* **270**(6 Pt 1)**:** L941-946.

Poulos, TL (2006) Soluble guanylate cyclase. *Curr Opin Struct Biol* **16**(6)**:** 736-743.

Priestley, J, Hey, W (1772) Observations on different kinds of air. *Philosophical Transactions***:** 147-264.

Radi, R, Rubbo, H, Bush, K, Freeman, BA (1997) Xanthine Oxidase Binding to Glycosaminoglycans: Kinetics and Superoxide Dismutase Interactions of Immobilized Xanthine Oxidase–Heparin Complexes. *Archives of Biochemistry and Biophysics* **339**(1)**:** 125-135.

Radomski, MW, Moncada, S (1993) Regulation of vascular homeostasis by nitric oxide. *Thromb Haemost* **70**(1)**:** 36-41.

Radomski, MW, Palmer, RM, Moncada, S (1990) Characterization of the L-arginine:nitric oxide pathway in human platelets. *Br J Pharmacol* **101**(2)**:** 325-328.

Rao, RM, Yang, L, Garcia-Cardena, G, Luscinskas, FW (2007) Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. *Circ Res* **101**(3)**:** 234-247.

Rawal, N, Pangburn, MK (1998) C5 convertase of the alternative pathway of complement. Kinetic analysis of the free and surface-bound forms of the enzyme. *J Biol Chem* **273**(27)**:** 16828-16835.

Reddy, KG, Nair, RN, Sheehan, HM, Hodgson, JM (1994) Evidence that selective endothelial dysfunction may occur in the absence of angiographic or ultrasound atherosclerosis in patients with risk factors for atherosclerosis. *J Am Coll Cardiol* **23**(4)**:** 833-843.

Richert, DA, Westerfeld, WW (1954) The relationship of iron to xanthine oxidase. *J Biol Chem* **209**(1)**:** 179-189.

Rikitake, Y, Kawashima, S, Takeshita, S, Yamashita, T, Azumi, H, Yasuhara, M, Nishi, H, Inoue, N, Yokoyama, M (2001) Anti-oxidative properties of fluvastatin, an HMG-CoA reductase inhibitor, contribute to prevention of atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **154**(1)**:** 87-96.

Roberts, AC, Porter, KE (2013) Cellular and molecular mechanisms of endothelial dysfunction in diabetes. *Diab Vasc Dis Res* **10**(6)**:** 472-482.

Rosenfeld, ME, Campbell, LA (2011) Pathogens and atherosclerosis: update on the potential contribution of multiple infectious organisms to the pathogenesis of atherosclerosis. *Thromb Haemost* **106**(5)**:** 858-867.

Rouquette, M, Page, S, Bryant, R, Benboubetra, M, Stevens, CR, Blake, DR, Whish, WD, Harrison, R, Tosh, D (1998) Xanthine oxidoreductase is asymmetrically localised on the outer surface of human endothelial and epithelial cells in culture. *FEBS Letters* **426**(3)**:** 397-401.

Rukoyatkina, N, Walter, U, Friebe, A, Gambaryan, S (2011) Differentiation of cGMP-dependent and -independent nitric oxide effects on platelet apoptosis and reactive oxygen species production using platelets lacking soluble guanylyl cyclase. *Thromb Haemost* **106**(5)**:** 922-933.

Sagar, S, Kallo, IJ, Kaul, N, Ganguly, NK, Sharma, BK (1992) Oxygen free radicals in essential hypertension. *Mol Cell Biochem* **111**(1-2)**:** 103-108.

Samal, AA, Honavar, J, Brandon, A, Bradley, KM, Doran, S, Liu, Y, Dunaway, C, Steele, C, Postlethwait, EM, Squadrito, GL, Fanucchi, MV, Matalon, S, Patel, RP (2012) Administration of nitrite after chlorine gas exposure prevents lung injury: effect of administration modality. *Free Radic Biol Med* **53**(7)**:** 1431-1439.

Sánchez-Lozada, LG, Tapia, E, Soto, V, Ávila-Casado, C, Franco, M, Zhao, L, Johnson, RJ (2008) Treatment with the xanthine oxidase inhibitor febuxostat lowers uric acid and alleviates systemic and glomerular hypertension in experimental hyperuricaemia. *Nephrology Dialysis Transplantation* **23**(4)**:** 1179-1185.

Saye, JA, Singer, HA, Peach, MJ (1984) Role of endothelium in conversion of angiotensin I to angiotensin II in rabbit aorta. *Hypertension* **6**(2 Pt 1)**:** 216-221.

Schardinger, RZ (1902) *Untersuch Nahrungs Genusmittel* **5:** 1113-1121.

Schiffrin, EL, Park, JB, Intengan, HD, Touyz, RM (2000) Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan. *Circulation* **101**(14)**:** 1653-1659.

Scotland, RS, Madhani, M, Chauhan, S, Moncada, S, Andresen, J, Nilsson, H, Hobbs, AJ, Ahluwalia, A (2005) Investigation of vascular responses in endothelial nitric oxide synthase/cyclooxygenase-1 double-knockout mice: key role for endothelium-derived hyperpolarizing factor in the regulation of blood pressure in vivo. *Circulation* **111**(6)**:** 796-803.

Sessa, WC, Pritchard, K, Seyedi, N, Wang, J, Hintze, TH (1994) Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ Res* **74**(2)**:** 349-353.

Shankar, A, Klein, BE, Klein, R (2004) Relationship between white blood cell count and incident hypertension. *Am J Hypertens* **17**(3)**:** 233-239.

Sharma, VS, Magde, D (1999) Activation of soluble guanylate cyclase by carbon monoxide and nitric oxide: a mechanistic model. *Methods* **19**(4)**:** 494-505.

Shi, C, Pamer, EG (2011) Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* **11**(11)**:** 762-774.

Shingles, R, Roh, MH, McCarty, RE (1997) Direct measurement of nitrite transport across erythrocyte membrane vesicles using the fluorescent probe, 6-methoxy-N-(3-sulfopropyl) quinolinium. *J Bioenerg Biomembr* **29**(6)**:** 611-616.

Shiva, S, Wang, X, Ringwood, LA, Xu, X, Yuditskaya, S, Annavajjhala, V, Miyajima, H, Hogg, N, Harris, ZL, Gladwin, MT (2006) Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nat Chem Biol* **2**(9)**:** 486-493.

Sindelar, JJ, Milkowski, AL (2012) Human safety controversies surrounding nitrate and nitrite in the diet. *Nitric Oxide* **26**(4)**:** 259-266.

Smith, CW, Marlin, SD, Rothlein, R, Toman, C, Anderson, DC (1989) Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *J Clin Invest* **83**(6)**:** 2008-2017.

Sozeri, B, Deveci, M, Dincel, N, Mir, S (2012) The early cardiovascular changes in pediatric patients with systemic lupus erythematosus. *Pediatr Nephrol* **28**(3)**:** 471-476.

Sparacino-Watkins, C, Tejero, J, Wang, J, Ragireddy, VP, Gauthier, M, Thomas, J, Bueno, M, Azarov, I, Lai, YC, Sun, B, Frizzell, S, Basu, P, Mora, A, Gladwin, MT (2013) Novel human mitochondrial enzyme can transform nitrite into nitric oxide. *Nitric Oxide-Biology and Chemistry* **31:** S45-S46.

Spiegelhalder, B, Eisenbrand, G, Preussmann, R (1976) Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in vivo formation of N-nitroso compounds. *Food Cosmet Toxicol* **14**(6)**:** 545-548.

Spiekermann, S, Landmesser, U, Dikalov, S, Bredt, M, Gamez, G, Tatge, H, Reepschlager, N, Hornig, B, Drexler, H, Harrison, DG (2003a) Electron spin resonance characterization of vascular xanthine and NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. *Circulation* **107**(10)**:** 1383-1389.

Spiekermann, S, Landmesser, U, Dikalov, S, Bredt, M, Gamez, G, Tatge, H, Reepschlager, N, Hornig, B, Drexler, H, Harrison, DG (2003b) Electron Spin Resonance Characterization of Vascular Xanthine and NAD(P)H Oxidase Activity in Patients With Coronary Artery Disease: Relation to Endothelium-Dependent Vasodilation. *Circulation* **107:** 1383-1389.

Springer, TA (1990) Adhesion receptors of the immune system. *Nature* **346**(6283)**:** 425-434.

Springer, TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**(2)**:** 301-314.

Stamler, J, Mendelsohn, ME, Amarante, P, Smick, D, Andon, N, Davies, PF, Cooke, JP, Loscalzo, J (1989) N-acetylcysteine potentiates platelet inhibition by endothelium-derived relaxing factor. *Circ Res* **65**(3)**:** 789-795.

Stamler, JS, Feelisch, M (1996) *Methods in nitric oxide research*. John Wiley and Sons: Chichester.

Stirpe, F, Della Corte, E (1969) The regulation of rat liver xanthine oxidase. Conversion in vitro of the enzyme activity from dehydrogenase (type D) to oxidase (type O). *J Biol Chem* **244**(14)**:** 3855-3863.

Stockert, AL, Shinde, SS, Anderson, RF, Hille, R (2002) The reaction mechanism of xanthine oxidase: evidence for two-electron chemistry rather than sequential one-electron steps. *J Am Chem Soc* **124**(49)**:** 14554-14555.

Stojanovic, A, Marjanovic, JA, Brovkovych, VM, Peng, X, Hay, N, Skidgel, RA, Du, X (2006) A phosphoinositide 3-kinase-AKT-nitric oxide-cGMP signaling pathway in stimulating platelet secretion and aggregation. *J Biol Chem* **281**(24)**:** 16333-16339.

Stokes, KY, Dugas, TR, Tang, Y, Garg, H, Guidry, E, Bryan, NS (2009) Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction. *Am J Physiol Heart Circ Physiol* **296**(5)**:** H1281-1288.

Stone, JR, Marletta, MA (1994) Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* **33**(18)**:** 5636-5640.

Stone, JR, Marletta, MA (1996) Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. *Biochemistry* **35**(4)**:** 1093-1099.

Sullivan, JC, Bhatia, K, Yamamoto, T, Elmarakby, AA (2010) Angiotensin (1-7) receptor antagonism equalizes angiotensin II-induced hypertension in male and female spontaneously hypertensive rats. *Hypertension* **56**(4)**:** 658-666.

Surdacki, A, Nowicki, M, Sandmann, J, Tsikas, D, Boeger, RH, Bode-Boeger, SM, Kruszelnicka-Kwiatkowska, O, Kokot, F, Dubiel, JS, Froelich, JC (1999) Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension. *J Cardiovasc Pharmacol* **33**(4)**:** 652-658.

Susin, J, Kmecl, V, Gregorcic, A (2006) A survey of nitrate and nitrite content of fruit and vegetables grown in Slovenia during 1996-2002. *Food Addit Contam* **23**(4)**:** 385-390.

Suzuki, H, DeLano, FA, Parks, DA, Jamshidi, N, Granger, DN, Ishii, H, Suematsu, M, Zweifach, BW, Schmid-Schonbein, GW (1998a) Xanthine oxidase activity associated with arterial blood pressure in spontaneously hypertensive rats. *Proc Natl Acad Sci U S A* **95**(8)**:** 4754-4759.

Suzuki, H, DeLano, FA, Parks, DA, Jamshidi, N, Granger, DN, Ishii, H, Suematsu, M, Zweifach, BW, Schmid-Schönbein, GW (1998b) Xanthine oxidase activity associated with arterial blood pressure in spontaneously hypertensive rats. *Proceedings of the National Academy of Sciences* **95**(8)**:** 4754-4759.

Svensson, S, Kjellgren, KI, Ahlner, J, Saljo, R (2000) Reasons for adherence with antihypertensive medication. *Int J Cardiol* **76**(2-3)**:** 157-163.

Szabo, C, Ischiropoulos, H, Radi, R (2007) Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nat Rev Drug Discov* **6**(8)**:** 662-680.

Szasz, T, Davis, RP, Garver, HS, Burnett, RJ, Fink, GD, Watts, SW (2013) Long-Term Inhibition of Xanthine Oxidase by Febuxostat Does Not Decrease Blood Pressure in Deoxycorticosterone Acetate (DOCA)-Salt Hypertensive Rats. *PLoS ONE* **8**(2)**:** e56046.

Taddei, S, Mattei, P, Virdis, A, Sudano, I, Ghiadoni, L, Salvetti, A (1994) Effect of potassium on vasodilation to acetylcholine in essential hypertension. *Hypertension* **23**(4)**:** 485-490.

Taddei, S, Virdis, A, Ghiadoni, L, Magagna, A, Salvetti, A (1998) Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation* **97**(22)**:** 2222-2229.

Tan, S, Yokoyama, Y, Dickens, E, Cash, TG, Freeman, BA, Parks, DA (1993) Xanthine oxidase activity in the circulation of rats following hemorrhagic shock. *Free Radic.Biol Med* **15**(4)**:** 407-414.

Tannenbaum, SR, Weisman, M, Fett, D (1976) The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet Toxicol* **14**(6)**:** 549-552.

Tayar, JH, Lopez-Olivo, MA, Suarez-Almazor, ME (2012) Febuxostat for treating chronic gout. *The Cochrane database of systematic reviews* **11:** CD008653.

Tedder, TF, Steeber, DA, Chen, A, Engel, P (1995) The selectins: vascular adhesion molecules. *FASEB J* **9**(10)**:** 866-873.

Terada, LS, Dormish, JJ, Shanley, PF, Leff, JA, Anderson, BO, Repine, JE (1992a) Circulating xanthine oxidase mediates lung neutrophil sequestration after intestinal ischemia-reperfusion. *Am J Physiol* **263**(3 Pt 1)**:** L394-L401.

Terada, LS, Dormish, JJ, Shanley, PF, Leff, JA, Anderson, BO, Repine, JE (1992b) Circulating xanthine oxidase mediates lung neutrophil sequestration after intestinal ischemia-reperfusion. *Am J Physiol* **263**(3 Pt 1)**:** L394-401.

Thomas, DD, Miranda, KM, Colton, CA, Citrin, D, Espey, MG, Wink, DA (2003) Heme proteins and nitric oxide (NO): the neglected, eloquent chemistry in NO redox signaling and regulation. *Antioxid Redox Signal* **5**(3)**:** 307-317.

Thomas, DD, Ridnour, LA, Isenberg, JS, Flores-Santana, W, Switzer, CH, Donzelli, S, Hussain, P, Vecoli, C, Paolocci, N, Ambs, S, Colton, CA, Harris, CC, Roberts, DD, Wink, DA (2008) The chemical biology of nitric oxide: implications in cellular signaling. *Free Radic Biol Med* **45**(1)**:** 18-31.

Thompson, HL, Matsushima, K (1992) Human polymorphonuclear leucocytes stimulated by tumour necrosis factor-alpha show increased adherence to extracellular matrix proteins which is mediated via the CD11b/18 complex. *Clin Exp Immunol* **90**(2)**:** 280-285.

Thomson, IA, Egginton, S, Hudlicka, O, Sims, MH (1994) Iloprost reduces leukocyte adhesion in skeletal muscle venules following ischaemia in a rat model of femorodistal bypass. *Eur J Vasc Surg* **8**(3)**:** 335-341.

Ting, HH, Timimi, FK, Boles, KS, Creager, SJ, Ganz, P, Creager, MA (1996) Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest* **97**(1)**:** 22-28.

Ting, HH, Timimi, FK, Haley, EA, Roddy, MA, Ganz, P, Creager, MA (1997) Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia. *Circulation* **95**(12)**:** 2617-2622.

Tiso, M, Tejero, J, Basu, S, Azarov, I, Wang, X, Simplaceanu, V, Frizzell, S, Jayaraman, T, Geary, L, Shapiro, C, Ho, C, Shiva, S, Kim-Shapiro, DB, Gladwin, MT (2011) Human neuroglobin functions as a redox-regulated nitrite reductase. *J Biol Chem* **286**(20)**:** 18277-18289.

Touyz, RM, Endemann, D, He, G, Li, JS, Schiffrin, EL (1999) Role of AT2 receptors in angiotensin II-stimulated contraction of small mesenteric arteries in young SHR. *Hypertension* **33**(1 Pt 2)**:** 366-372.

Townsend, N, Wickramasinghe, K, Bhatnagar, P, Smolina, K, Nichols, M, Leal, J, Luengo-Fernandez, R, Rayner, M (2012) Coronary heart disease statisitics: *A compendium of health statistics*. *British Heart Foundation*.

Tripatara, P, Patel, NS, Webb, A, Rathod, K, Lecomte, FM, Mazzon, E, Cuzzocrea, S, Yaqoob, MM, Ahluwalia, A, Thiemermann, C (2007) Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: role for xanthine oxidoreductase. *J Am Soc Nephrol* **18**(2)**:** 570-580.

Tsikas, D (2007a) Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. *J Chromatogr.B Analyt.Technol.Biomed.Life Sci.* **851**(1-2)**:** 51-70.

Tsikas, D (2007b) Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. *J Chromatogr B Analyt Technol Biomed Life Sci* **851**(1-2)**:** 51-70.

van Leeuwen, M, Gijbels, MJ, Duijvestijn, A, Smook, M, van de Gaar, MJ, Heeringa, P, de Winther, MP, Tervaert, JW (2008) Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR-/- mice. *Arterioscler Thromb Vasc Biol* **28**(1)**:** 84-89.

van Velzen, AG, Sips, AJ, Schothorst, RC, Lambers, AC, Meulenbelt, J (2008) The oral bioavailability of nitrate from nitrate-rich vegetables in humans. *Toxicol Lett* **181**(3)**:** 177-181.

Vanizor, B, Orem, A, Karahan, SC, Kiran, E, Erem, C, Aliyazicioglu, R, Uydu, HA (2001) Decreased nitric oxide end-products and its relationship with high density lipoprotein and oxidative stress in people with type 2 diabetes without complications. *Diabetes Res Clin Pract* **54**(1)**:** 33-39.

Vasquez-Vivar, J, Kalyanaraman, B, Martasek, P, Hogg, N, Masters, BS, Karoui, H, Tordo, P, Pritchard, KA, Jr. (1998) Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A* **95**(16)**:** 9220-9225.

Velmurugan, S, Kapil, V, Ghosh, SM, Davies, S, McKnight, A, Aboud, Z, Khambata, RS, Webb, AJ, Poole, A, Ahluwalia, A (2013) Antiplatelet effects of dietary nitrate in healthy volunteers: involvement of cGMP and influence of sex. *Free Radic Biol Med* **65:** 1521-1532.

Villar, IC, Francis, S, Webb, A, Hobbs, AJ, Ahluwalia, A (2006) Novel aspects of endothelium-dependent regulation of vascular tone. *Kidney Int* **70**(5)**:** 840-853.

Villar, IC, Scotland, RS, Khambata, RS, Chan, M, Duchene, J, Sampaio, AL, Perretti, M, Hobbs, AJ, Ahluwalia, A (2011) Suppression of endothelial P-selectin expression contributes to reduced cell trafficking in females: an effect independent of NO and prostacyclin. *Arterioscler Thromb Vasc Biol* **31**(5)**:** 1075-1083.

Vleeming, W, van de Kuil, A, te Biesebeek, JD, Meulenbelt, J, Boink, AB (1997a) Effect of nitrite on blood pressure in anaesthetized and free-moving rats. *Food Chem Toxicol* **35**(6)**:** 615-619.

Vleeming, W, Van De Kuil, A, te Biesebeek, JD, Meulenbelt, J, Boink, ABTJ (1997b) Effect of nitrite on blood pressure in anaesthetized and free-moving rats. *Food and Chemical Toxicology* **35:** 615-619.

von Andrian, UH, Chambers, JD, McEvoy, LM, Bargatze, RF, Arfors, KE, Butcher, EC (1991) Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo. *Proc Natl Acad Sci U S A* **88**(17)**:** 7538-7542.

Wagner, DA, Schultz, DS, Deen, WM, Young, VR, Tannenbaum, SR (1983) Metabolic fate of an oral dose of 15N-labeled nitrate in humans: effect of diet supplementation with ascorbic acid. *Cancer Res* **43**(4)**:** 1921-1925.

Wallwork, CJ, Parks, DA, Schmid-Schönbein, GW (2003) Xanthine oxidase activity in the dexamethasone-induced hypertensive rat. *Microvascular Research* **66**(1)**:** 30-37.

Wang, D, Strandgaard, S, Iversen, J, Wilcox, CS (2009) Asymmetric dimethylarginine, oxidative stress, and vascular nitric oxide synthase in essential hypertension. *Am J Physiol Regul Integr Comp Physiol* **296**(2)**:** R195-200.

Wang, J, Krizowski, S, Fischer, K, Niks, D, Tejero, J, Wang, L, Sparacino-Watkins, C, Ragireddy, P, Frizzell, S, Kelley, EE, Shiva, S, Zhang, YZ, Basu, P, Hille, R, Schwarz, G, Gladwin, MT (2013) Sulfite oxidase catalyzes single electron transfer at molybdenum domain to reduce nitrite to NO. *Nitric Oxide-Biology and Chemistry* **31:** S39-S40.

Webb, A, Bond, R, McLean, P, Uppal, R, Benjamin, N, Ahluwalia, A (2004a) Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc Natl Acad Sci U S A* **101**(37)**:** 13683-13688.

Webb, A, Bond, R, McLean, P, Uppal, R, Benjamin, N, Ahluwalia, A (2004b) Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc.Natl.Acad.Sci.U.S.A* **101**(37)**:** 13683-13688.

Webb, AJ, Milsom, AB, Rathod, KS, Chu, WL, Qureshi, S, Lovell, MJ, Lecomte, FM, Perrett, D, Raimondo, C, Khoshbin, E, Ahmed, Z, Uppal, R, Benjamin, N, Hobbs, AJ, Ahluwalia, A (2008a) Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase. *Circ Res* **103**(9)**:** 957-964.

Webb, AJ, Milsom, AB, Rathod, KS, Chu, WL, Qureshi, S, Lovell, MJ, Lecomte, FM, Perrett, D, Raimondo, C, Khoshbin, E, Ahmed, Z, Uppal, R, Benjamin, N, Hobbs, AJ, Ahluwalia, A (2008b) Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase. *Circulation Research* **103**(9)**:** 957-964.

Webb, AJ, Patel, N, Loukogeorgakis, S, Okorie, M, Aboud, Z, Misra, S, Rashid, R, Miall, P, Deanfield, J, Benjamin, N, MacAllister, R, Hobbs, AJ, Ahluwalia, A (2008c) Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension* **51**(3)**:** 784-790.

Weber, C, Noels, H (2011) Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med* **17**(11)**:** 1410-1422.

Weber, C, Zernecke, A, Libby, P (2008) The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol* **8**(10)**:** 802-815.

Wedel, B, Harteneck, C, Foerster, J, Friebe, A, Schultz, G, Koesling, D (1995) Functional domains of soluble guanylyl cyclase. *J Biol Chem* **270**(42)**:** 24871-24875.

Wennmalm, A, Benthin, G, Edlund, A, Jungersten, L, Kieler-Jensen, N, Lundin, S, Westfelt, UN, Petersson, AS, Waagstein, F (1993) Metabolism and excretion of nitric oxide in humans. An experimental and clinical study. *Circ Res* **73**(6)**:** 1121-1127.

White, CR, Darley-Usmar, V, Berrington, WR, McAdams, M, Gore, JZ, Thompson, JA, Parks, DA, Tarpey, MM, Freeman, BA (1996a) Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc.Natl.Acad.Sci.U.S.A* **93**(16)**:** 8745-8749.

White, CR, Darley-Usmar, V, Berrington, WR, McAdams, M, Gore, JZ, Thompson, JA, Parks, DA, Tarpey, MM, Freeman, BA (1996b) Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc Natl Acad Sci U S A* **93**(16)**:** 8745-8749.

WHO (2010) Global status report on noncommunicable diseases 2010.

Williams, B, Poulter, NR, Brown, MJ, Davis, M, McInnes, GT, Potter, JF, Sever, PS, Mc, GTS (2004) Guidelines for management of hypertension: report of the fourth working party of the British Hypertension Society, 2004-BHS IV. *J Hum Hypertens* **18**(3)**:** 139-185.

Williamson, JR, Schaffer, SW, Ford, C, Safer, B (1976) Contribution of tissue acidosis to ischemic injury in the perfused rat heart. *Circulation* **53**(3 Suppl)**:** I3-14.

Wilson, AM, Harada, R, Nair, N, Balasubramanian, N, Cooke, JP (2007) L-arginine supplementation in peripheral arterial disease: no benefit and possible harm. *Circulation* **116**(2)**:** 188-195.

Wink, DA, Cook, JA, Kim, SY, Vodovotz, Y, Pacelli, R, Krishna, MC, Russo, A, Mitchell, JB, Jourd'heuil, D, Miles, AM, Grisham, MB (1997) Superoxide modulates the oxidation and nitrosation of thiols by nitric oxide-derived reactive intermediates. Chemical

aspects involved in the balance between oxidative and nitrosative stress. *J Biol Chem* **272**(17)**:** 11147-11151.

Wink, DA, Hanbauer, I, Grisham, MB, Laval, F, Nims, RW, Laval, J, Cook, J, Pacelli, R, Liebmann, J, Krishna, M, Ford, PC, Mitchell, JB (1996) Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. *Curr Top Cell Regul* **34:** 159-187.

Won, D, Zhu, SN, Chen, M, Teichert, AM, Fish, JE, Matouk, CC, Bonert, M, Ojha, M, Marsden, PA, Cybulsky, MI (2007) Relative reduction of endothelial nitric-oxide synthase expression and transcription in atherosclerosis-prone regions of the mouse aorta and in an in vitro model of disturbed flow. *Am J Pathol* **171**(5)**:** 1691-1704.

Yasuda, M, Takeuchi, K, Hiruma, M, Iida, H, Tahara, A, Itagane, H, Toda, I, Akioka, K, Teragaki, M, Oku, H, et al. (1990) The complement system in ischemic heart disease. *Circulation* **81**(1)**:** 156-163.

Yokoyama, Y, Beckman, JS, Beckman, TK, Wheat, JK, Cash, TG, Freeman, BA, Parks, DA (1990) Circulating xanthine oxidase: potential mediator of ischemic injury. *Am J Physiol* **258**(4 Pt 1)**:** G564-G570.

Zernecke, A, Bot, I, Djalali-Talab, Y, Shagdarsuren, E, Bidzhekov, K, Meiler, S, Krohn, R, Schober, A, Sperandio, M, Soehnlein, O, Bornemann, J, Tacke, F, Biessen, EA, Weber, C (2008) Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. *Circ Res* **102**(2)**:** 209-217.

Zhang, G, Xiang, B, Dong, A, Skoda, RC, Daugherty, A, Smyth, SS, Du, X, Li, Z (2011) Biphasic roles for soluble guanylyl cyclase (sGC) in platelet activation. *Blood* **118**(13)**:** 3670-3679.

Zhang, Z, Naughton, D, Winyard, PG, Benjamin, N, Blake, DR, Symons, MC (1998a) Generation of nitric oxide by a nitrite reductase activity of xanthine oxidase: a

potential pathway for nitric oxide formation in the absence of nitric oxide synthase activity. *Biochem Biophys Res Commun* **249**(3)**:** 767-772.

Zhang, Z, Naughton, D, Winyard, PG, Benjamin, N, Blake, DR, Symons, MC (1998b) Generation of nitric oxide by a nitrite reductase activity of xanthine oxidase: a potential pathway for nitric oxide formation in the absence of nitric oxide synthase activity. *Biochem.Biophys.Res Commun.* **249**(3)**:** 767-772.

Zhang, Z, Naughton, DP, Blake, DR, Benjamin, N, Stevens, CR, Winyard, PG, Symons, MC, Harrison, R (1997) Human xanthine oxidase converts nitrite ions into nitric oxide (NO). *Biochem.Soc.Trans.* **25**(3)**:** 524S.

Zhao, Y, Brandish, PE, Ballou, DP, Marletta, MA (1999) A molecular basis for nitric oxide sensing by soluble guanylate cyclase. *Proc Natl Acad Sci U S A* **96**(26)**:** 14753-14758.

Zineh, I, Beitelshees, AL, Welder, GJ, Hou, W, Chegini, N, Wu, J, Cresci, S, Province, MA, Spertus, JA (2008) Epithelial neutrophil-activating peptide (ENA-78), acute coronary syndrome prognosis, and modulatory effect of statins. *PLoS One* **3**(9)**:** e3117.

Zweier, JL, Samouilov, A, Kuppusamy, P (1999) Non-enzymatic nitric oxide synthesis in biological systems. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1411**(2–3)**:** 250-262.

Zweier, JL, Wang, P, Samouilov, A, Kuppusamy, P (1995) Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med* **1**(8)**:** 804-809.